MACROPHAGE-STIMULATING PROTEIN / RON IS A NOVEL MEDIATOR OF OSTEOCLAST ACTIVATION PROMOTING BREAST CANCER-INDUCED BONE DESTRUCTION AND OSTEOPOROSIS

by

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STATEMENT OF DISSERTATION APPROVAL

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ABSTRACT

The purpose of this dissertation is to define how the macrophage-stimulating protein (MSP) / Ron receptor tyrosine kinase pathway is involved in breast cancer-induced bone destruction and osteoclast activation. These studies employed *in vitro* osteoclast cultures and *in vivo* animal tumor models as systems to explore the ability of MSP to stimulate osteoclast activity and the ability of MSP-expressing tumor cells to cause bone destruction. I have also explored signaling downstream of Ron in osteoclasts and the requirement for Ron in bone destruction. I have utilized both genetic and pharmacological methods to target this pathway, thereby rigorously testing the ability of this pathway to activate osteoclasts.

Included in the first part of this dissertation are two reviews. The first describes the MSP/Ron pathway, its function in cancer and inflammation, and the potential for targeting this pathway pharmacologically. The second review describes various mouse models used to study bone metastasis and bone destruction *in vivo.*

The second part of this dissertation is focused on the ability of MSP/Ron to regulate osteoclast activation. Tumors overexpressing MSP spontaneously metastasize to bone and are osteolytic in an animal model of breast cancer. Here, I explore the function of MSP/Ron in aiding cancer cells to manipulate the

bone microenvironment, allowing for tumor growth and bone destruction. This study reveals the MSP/Ron pathway as a novel mediator of osteoclast activity, independent of previously established pathways such as RANKL and TGFβ. Additionally, I have demonstrated that the loss or inhibition of the MSP/Ron pathway can protect from bone loss due to both breast cancer and osteoporosis. This evidence establishes the importance of this pathway in osteoclast regulation and the potential for use of Ron inhibitors in preventing bone loss.

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point of view and very rarely get to directly experience the impact that research can have on a person's life. These experiences greatly influenced me and my thinking in terms of career perspectives for the future.

CHAPTER 1

INTRODUCTION

1.1 Bone remodeling

The skeleton serves many functions, including structural support, maintenance of mineral homeostasis, as a reservoir of growth factors and cytokines, and it provides the environment for hematopoiesis within the marrow spaces (Taichman 2005). Bone is a highly dynamic organ that is continuously molded, shaped, and repaired. The microarchitecture of bone is developed to provide maximal strength with minimal mass according to the physiological needs of the organism. Once formed, bone undergoes a process termed remodeling that involves destruction (resorption) and building of new bone (synthesis), which occurs throughout the skeleton. In adults, approximately 10% of the skeleton is remodeled per year with the entire skeleton replaced every 10 years. Remodeling is crucial to remove old, microdamaged bone and replace it with new, mechanically stronger bone to help preserve bone strength. The development and activation of osteoclasts is an essential process for skeletal growth during bone and mineral homeostasis. Homeostasis involves the removal of mineralized bone by osteoclasts and the formation and subsequent

mineralization of bone through the action of osteoblasts. Imbalances in remodeling can result in perturbations of skeletal structure and function. Many skeletal diseases are due to excess osteoclastic activity, leading to a net loss of bone mass and thereby increasing skeletal fragility and the risk of fracture. Such diseases include osteoporosis, osteolytic tumor metastases, rheumatoid arthritis, and multiple myeloma.

1.2 Osteoclast function and lifecycle

Osteoclasts are multinucleated cells derived from hematopoietic precursors of the monocyte-macrophage lineage. They are responsible for the degradation of mineralized bone matrix during organism development and growth, and for skeletal homeostasis, repair, and bone remodeling throughout life (Figure 1.1). Osteoclastic resorption is regulated by the rate of differentiation of osteoclasts from monocyte precursors, the activity level of mature osteoclasts, and the survival time of the mature cells (Baron 2011). The discovery of two key cytokines, sufficient to induce osteoclast differentiation, allowed a greater understanding of the regulation of osteoclastogenesis (Figure 1.2). Macrophage colony-stimulating factor (M-CSF) binds to its receptor c-Fms, leading to proliferation and survival of monocyte precursor cells. RANKL binds to its receptor RANK, and together with M-CSF initiates commitment and progression of early precursors to the osteoclast lineage (Asagiri 2007; Boyle 2003; Elford 1987; Lacey 1998; Yoshida 1990). Upon commitment to the osteoclast lineage, precursor cells fuse to form a polykaryon containing many nuclei. Osteoclast cell-

cell fusion is a crucial component of osteoclastogenesis and is important for the cytoskeletal reorganization that is required for bone resorption. While the exact mechanisms regulating osteoclast precursor fusion remain largely unknown, one key factor involved is dendritic cell-specific transmembrane protein (DC-STAMP). Originally identified in dendritic cells, DC-STAMP is also expressed in macrophages and osteoclasts. A knockout animal model of DC-STAMP results in little to no fusion of osteoclasts, demonstrating the necessity of this factor in the fusion process (Yagi 2005). Other factors involved in the fusion process are Atp6v0d2, OC-STAMP, and CD9 (Ishii 2006; Lee 2006; Miyamoto 2012). Expression of these factors is largely dependent upon the key transcription factors NFATc1 and c-Fos, in conjunction with MITF and PU.1 (Mellis 2011). While several components involved in osteoclastogenesis have been identified, much work remains to delineate the processes involved and the interactions between key contributors in multinucleated osteoclast formation.

There are many steps required for a mature osteoclast to actively resorb bone. Osteoclasts must first attach to the bone surface and migrate along the bone surface to the correct location. Next, they become polarized and form new specialized membrane domains. They then synthesize and secrete hydrolytic enzymes onto the bone matrix and acidify the resorption lacunae by using vacuolar proton pumps and chloride channels. Finally, they remove the extracellular bone matrix degradation products by internalization and transendocytosis of the matrix protein fragments (Baron 2011; Vaananen 2000). Each of these processes is described in detail below.

1.2.1 Formation of the sealing zone

After migration of the osteoclast to a resorption site, a specific membrane domain called the sealing zone or actin ring forms. The plasma membrane attaches tightly to the bone matrix and seals the resorption site from its surroundings, forming a resorption lacuna. This actin ring is composed of filamentous actin and densely packed podosomes, which consist of an actin core surrounded by cytoskeleton-regulating proteins such as vinculin, talin, paxilin, and cortactin (Zou 2010). While other molecules participate, the association of osteoclasts with bone matrix is primarily mediated by integrins. The αvβ3 integrin is highly expressed in osteoclasts and its cytoplasmic tail has been shown to recognize the RGD motif (Arg-Gly-Asp) contained in proteins residing within the bone matrix, such as osteopontin and bone sialic protein (Zou 2010). RGD-containing protein engagement wtih αvβ3 leads to c-Src activation within the osteoclast cytoplasm. Syk is then recruited to the integrin-stimulated signaling complex by Dap12. Along with the adaptor protein Slp-76, Syk phosphorylates the guanine nucleotide exchange factor Vav3, which converts the Rho guanine trinucelotide phosphatase (GTPase) Rac from its inactive (GDP-bound) to its active (GTP-bound) form. Rac then reorganizes the osteoclast cytoskeleton, leading to actin ring formation (Izawa 2012).

1.2.2 Bone resorption

Bone resorbing osteoclasts are highly polarized and contain distinct membrane domains: the “ruffled border,” a functional secretory domain, and a

basolateral membrane. After the formation of the actin ring, osteoclasts form a specialized resorbing organelle, the ruffled border, within the actin ring structure. The ruffled border is formed by the fusion of intracellular acidic vesicles with the region of plasma membrane facing the bone matrix. The secretion of protease-containing vesicles from the ruffled border onto the bone surface causes degradation of the collagen matrix and dissolution of hydroxyapatite mineral (Teitelbaum 2000). Several proteolytic enzymes are important for degrading the organic bone matrix, including lysosomal cysteine proteinases such as cathepsin K, and matrix metalloproteinases such as MMP9 and MMP13. In addition to degradation of the bone protein matrix, the dissolution of the crystalline hydroxyapatite within bone is achieved by targeted secretion of hydrochloric acid (HCl) into the resorption lacuna, through ATP-consuming vacuolar proton pumps. Protons for the proton pump are produced by cytoplasmic carbonic anhydrase II, while chloride channels in the ruffled border allow a flow of chloride anions into the resorption lacuna maintaining electroneutrality (Schlesinger 1997). After degradation, the byproducts are removed from the resorption lacuna through a transcytotic vesicular pathway from the ruffled border to the functional secretory domain where they are released into the extracellular space (Vaananen 2000).

1.2.3 Osteoclast survival

In order to maintain the critical balance between bone resorption and bone formation, osteoclasts undergo apoptosis at sites where new bone is laid down by osteoblasts. Although many factors can contribute to osteolclast lifespan,

estrogen is a key hormone that maintains bone mass, in part by promoting osteoclast apoptosis via regulating TGFβ and Fas ligand expression (Boyce 2013). Osteoclast survival, on the other hand, is enhanced by various cytokines through up-regulation of Ras/Rac1/Erk and PI3K/mTOR/S6K signaling (Tanaka 2006). Cytokine withdrawal leads to reduced expression of the anti-apoptotic protein, Bcl-2, and rapid apoptosis (Tanaka 2010).

1.3 RANKL/RANK signaling in bone remodeling

A breakthrough in the understanding of osteoclast differentiation and activation has come from the analysis of a family of biologically-related tumor necrosis factor (TNF) receptor/TNF-like proteins: osteoprotegerin (OPG), receptor activator of nuclear factor (NF)-kB (RANK), and RANK ligand (RANKL). These factors, along with M-CSF, are major players in the regulation of osteoclast function and survival. The RANKL polypeptide is a type II transmembrane protein found on the surface of expressing cells, but it also exists as a proteolytically released soluble form (Lacey 1998). Most hormones and factors that are known to stimulate bone resorption *in vivo* do so by inducing the expression of RANKL on stromal cells and osteoblasts. Activation of RANK on osteoclasts by RANKL leads to differentiation of osteoclast precursor cells, osteoclast activation, and survival. M-CSF, in conjunction with RANKL/RANK, is crucial for osteoclast differentiation and survival; one of the earliest effects of M-CSF is to promote expression of RANK by myeloid progenitors, which primes the cells to respond to RANKL (Boyce 2012).

RANKL has been described as a key coupling factor linking osteoclast and osteoblast functions, contributing to the normally balanced activities of these two cell types. RANKL expression by osteoblasts coordinates bone remodeling by stimulating the differentiation and bone resorptive activity of local osteoclasts, which in turn stimulate bone synthesis by closely adjacent osteoblasts (Udagawa 2000). While there are likely many coupling factors yet to be discovered, both semaphorins and ephrins have been implicated in the complex signaling between osteoblasts and osteoclasts, regulating their activity both positively and negatively (Sims 2014). However, there are other sources of RANKL aside from osteoblasts. During the process of bone formation, osteoblasts can become embedded within the bone matrix and differentiate into a specialized cell called the osteocyte. Osteocytes continue to live and function within the mineralized tissue by forming long dendrite-like extensions that are important for signaling and the exchange of nutrients and waste throughout the canaliculi and with cells in the marrow cavity. Moreover, osteocytes are thought to be mechanosensor cells that control the activity of osteoblasts and osteoclasts by influencing the balance of activity in these cell types depending on the mechanical loading of the bone (Xiong 2012). For example, osteocytes specifically express Sclerostin, which inhibits osteoblasts by antagonizing Wnt signaling, but also produce RANKL, resulting in increased osteoclastogenesis (Komori 2013). While their role in bone remodeling has historically been underappreciated, recent work has shed light on the crucial functions osteocytes play in the regulation of bone biology. Experiments utilizing osteoblast-specific ablation have demonstrated that

osteoblasts are not essential osteoclast support cells: ablation of osteoblasts had no effect on osteoclast surface or bone resorption markers and did not affect the basal RANKL mRNA levels in the bone (Corral 1998, Galli 2009). These results were surprising; the accepted model had been that osteoblasts were essential for regulating osteoclast function and did so through the production of RANKL. Osteocytes were also known to produce RANKL, but it was not thought that they produce levels that are high enough to significantly affect osteoclast activity. Definitive proof for the osteocyte as a crucial regulator of osteoclast function came from experiments performed using osteocyte-specific conditional deletion of RANKL in mice. Deletion of RANKL in osteocytes resulted in reduction of osteoclast numbers and markers of bone resorption, and an increase in bone mass with age. Importantly, the lack of RANKL in osteocytes had no effect on bone resorption during development, suggesting that osteocyte-derived RANKL specifically contributes to bone remodeling in adults (Nakashima 2011; Xiong 2011). These results strongly suggested that osteocytes, not osteoblasts, are an essential source of RANKL controlling osteoclast formation during cancellous bone remodeling. Regardless of the source, these experiments clearly established the importance of RANKL in regulating the function of osteoclasts.

OPG was originally discovered as a soluble protein produced by osteoblasts that could block osteoclast formation *in vitro* and bone resorption *in vivo*. Later, OPG was discovered to function as a decoy receptor that can block binding of RANKL to its cellular receptor, RANK. Expression of RANKL and OPG is normally coordinated, in order to regulate bone resorption and density both

positively and negatively by controlling the activation state of RANK on osteoclasts (Boyle 2003).

Activation of RANK by its ligand leads to expression of osteoclast-specific genes during differentiation, activation, and during survival. At least five distinct signaling cascades mediated by protein kinases are induced during osteoclastogenesis and activation: inhibitor of NF-kB kinase (IKK), c-Jun N-terminal kinase (JNK), p38 MAPK, extracellular signal-regulated kinase (ERK), and Src pathways. Like other TNF receptor family members, RANK lacks intrinsic kinase activity to phosphorylate and activate downstream signaling molecules. TNFR-associated cytoplasmic factor 6 (TRAF6) acts as a key adaptor allowing RANK to assemble signaling proteins that direct osteoclast-specific gene expression. For example, stimulation of p38 results in the downstream activation of the transcriptional regulator Mitf, which controls the expression of the genes encoding tartrate-resistant acid phosphatase (TRAP) and cathepsin K. Mitf, cathepsin K, and TRAP are all required by the mature osteoclast for bone resorption (Mansky 2002). RANK- and M-CSF-mediated signaling through PI3K and the serine/threonine protein kinase Akt act in cooperation with Src to induce cell survival, cytoskeletal rearrangements, and motility (Wong 1999). Finally, RANKL signaling through NF-kB p65 prevents Bid- and Caspase3-induced osteoclast apoptosis (Vaira 2008), while M-CSF prevents apoptosis by several mechanisms, including activation of Bcl-2 via Mitf, increased degradation of Bim by c-Cbl, and upregulation of Bcl-XL (Tanaka 2006; Tanaka 2010). The discovery of the RANK/RANKL and M-CSF signaling axes has advanced the understanding

of osteoclast function and its role in bone biology. In addition to these discoveries, however, RANKL-induced differentiation of osteoclasts has served as a useful tool for the discovery of a network of numerous other pathways regulating osteoclast function.

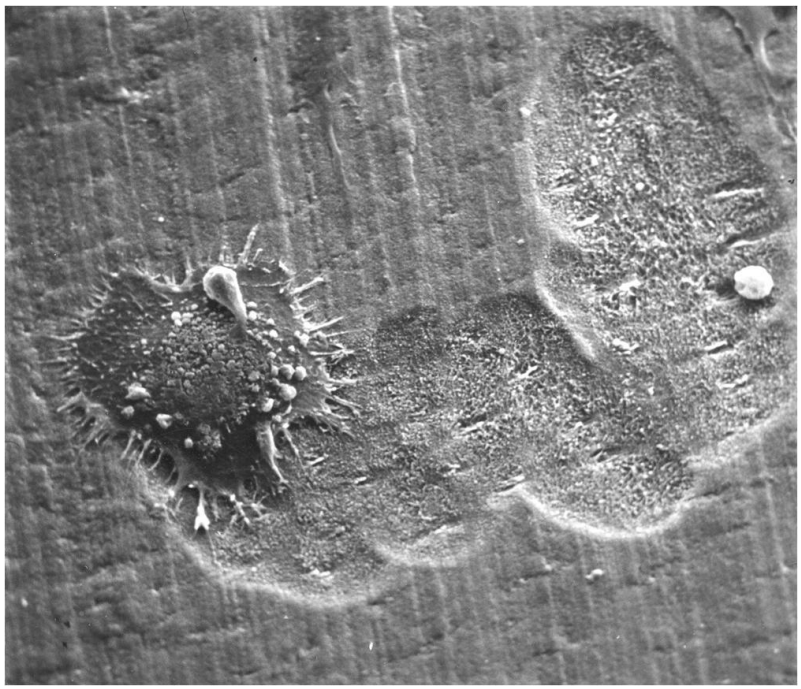
1.4 RANKL-independent mechanisms of bone remodeling

Despite the importance of M-CSF and RANKL in osteoclast function, several factors have been identified that can substitute for either of these factors under certain conditions or in specific osteoclast processes. The importance of M-CSF in osteoclast formation is evidenced by the fact that *op/op* osteopetrotic mice, which exhibit a spontaneous mutation in the M-CSF encoding gene (*Csf1*), have very few osteoclasts and markedly decreased bone resorption (Felix 1990; Yoshida 1990). However, the presence of some osteoclasts and the fact that op/op mice undergo a spontaneous age-dependent recovery of osteoclastogenesis suggested that there may be factors that can substitute for M-CSF function (Begg 1993; Felix 1994). Indeed, *in vitro* studies have demonstrated that there are several growth factors capable of regulating osteoclast function, at least to a certain degree, in the absence of M-CSF. Both Flt3 ligand and vascular endothelial growth factor (VEGF) have been shown to support osteoclastogenesis in the absence of functional M-CSF. Osteoclasts that formed upon addition of Flt3 or VEGF are multinucleated, express TRAP, and are capable of lacunar resorption. However, they were significantly smaller in size and their resorption was reduced compared to that of M-CSF stimulated

osteoclasts (Lean 2001;Niida 1999). Addition of hepatocyte growth factor (HGF) in lieu of M-CSF also results in differentiation and activation of osteoclasts *in vitro* (Adamopoulos 2006). Similar to that of Flt3 and VEGF stimulated osteoclasts, the HGF-stimulated osteoclasts are smaller compared to M-CSF-stimulated cells but were actually capable of significantly greater resorption than Flt3- or VEGF-stimulated cells. Similar results were also demonstrated with placental growth factor (PlGF), suggesting functional redundancy between several growth factors that are highly expressed in the bone marrow environment (Taylor 2012). The addition of these growth factors to osteoclasts stimulated with M-CSF and RANKL also results in significant increases in differentiation and activation, suggesting that growth factors may modulate these functions with the majority of regulation controlled by M-CSF. In addition, these growth factors may provide a salvage pathway for osteoclast formation in the absence of M-CSF, though the role these factors play under *in vivo* physiologic conditions has yet to be demonstrated (Taylor 2012).

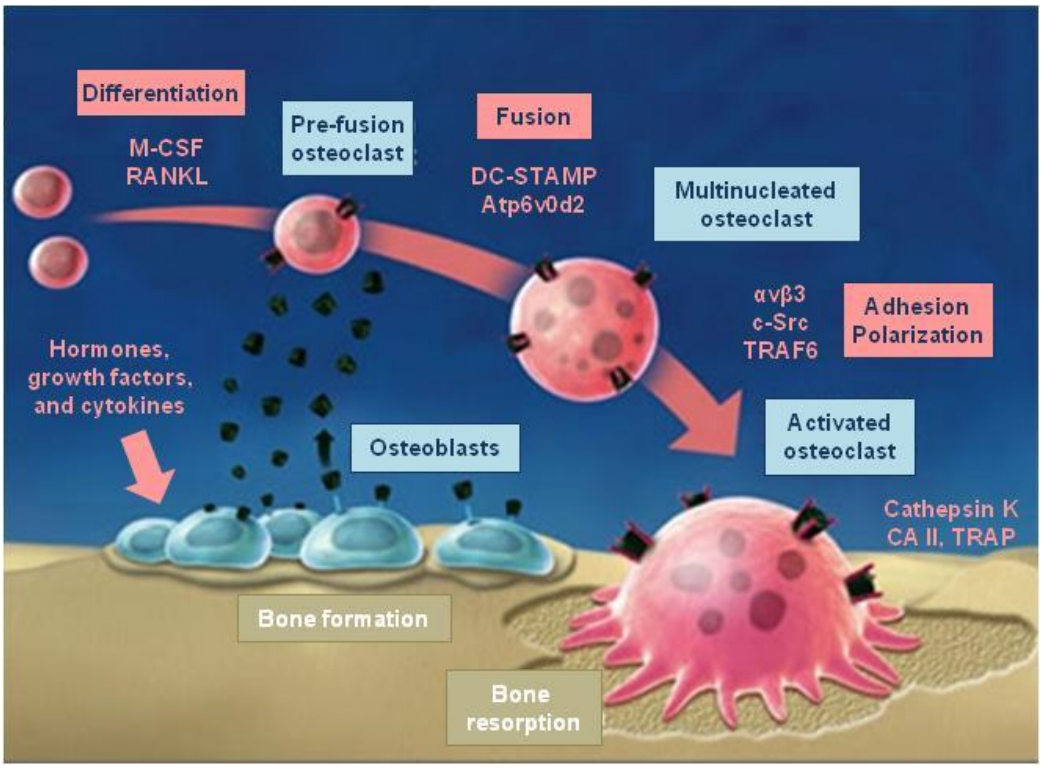
In addition to potential M-CSF-independent osteoclastogenesis mechanisms, several RANKL-independent mechanisms have also been discovered. Interleukin-6 (IL-6), interleukin-11 (IL-11), and tumor necrosis factor α (TNF-α) have all been shown to increase proliferation and differentiation of osteoclast precursor cells *in vitro*, independent of RANKL. These factors are also capable of stimulating a limited amount of resorption and, in the case of IL-1, are capable of prolonging osteoclast survival in the absence of RANKL (Fuller 2002; Kobayashi 2000; Kudo 2003). While the ability of these factors to perform these

functions under normal conditions *in vivo* is unclear, there is evidence for an important role for several of these cytokines in bone loss associated with pathologic diseases. For example, TNFα is crucial in the pathogenesis of bone and joint destruction that occurs in rheumatoid arthritis and other forms of chronic inflammatory osteolysis (Fuller 2002). It has also been reported that TNFα, IL-6, and IL-11 mediate bone loss associated with estrogen deficiency (Kimble 1996; Cenci 2000; Jilka 1992; Manolagas 1995). Finally, IL-6 has been implicated in the pathogenesis of osteolysis associated with Paget's disease, multiple myeloma, and Gorham-Stout disease (Kudo 2003). It is not clear, however, whether RANKL cooperates with these factors to regulate osteoclast activity in diseased conditions.



Alan Boyde, Bone Research Society

Figure 1.1 Scanning electron micrograph of an osteoclast actively resorbing bone.



Adapted from Zhenya Senyak, MPNforum.com

Figure 1.2 The lifecycle of an osteoclast. Maturation occurs on bone from peripheral blood-born mononuclear cells with traits of the macrophage lineage. M-CSF (CSF-1) and RANKL are essential for osteoclastogenesis and throughout the lifespan of an osteoclast. Exposure of mononuclear cells to M-CSF leads to proliferation and expression of the RANK receptor. RANKL, produced by osteoblasts and osteocytes, leads to cell-fate commitment and the expression of proteins responsible for fusion. Mononuclear osteoclast precursor cells then fuse, forming a multinuclear osteoclast containing approximately 10 to 20 nuclei. Further stimulation by M-CSF and RANKL leads to the expression of proteins required for the reorganization of the cytoskeleton, adhesion, and formation of the sealing zone. The fully mature, activated osteoclast then begins to produce the factors which degrade the bone, consisting of organic and inorganic matrix.

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CHAPTER 2

MOUSE MODELS OF BREAST CANCER METASTASIS TO BONE

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Mouse models of breast cancer metastasis to bone

Kelsi L. Kretschmann · Alana L. Welm

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Abstract Breast cancer frequently metastasizes to bone, where it takes a significant toll on quality of life. Models of bone metastasis are needed in order to better understand the process of bone metastasis and to develop better treatments. Here, we discuss the available mouse models for breast cancer bone metastasis and critical techniques for imaging bone metastasis in these models.

Keywords Breast cancer · Bone metastasis · Mouse model

1 Introduction

Breast cancer is the second leading cause of malignancy in American women with more than 200,000 new cases diagnosed each year [1]. Despite typically successful treatment of the primary malignancy, metastasis occurs at distant sites in about 30 % of patients, which results in a generally incurable disease. Bone is the most common site of metastasis, occurring in over 70 % of breast cancer patients with advanced disease [2]. The majority of bone metastasis from breast cancer is osteolytic, or bone destructive [3]. Complications associated with these lesions include pathologic fractures, severe pain, nerve compression, anemia, and hypercalcemia [4]. While it is clear that bone metastasis in breast cancer significantly impacts the quality of life for these patients and represents a serious public health problem, limited progress has been made in preventing or resolving bone metastasis. There has been some success with the use of bisphosphonates and the recent development of Denosumab, but treatment with these drugs is mostly palliative [4]. In order to develop new drugs that prevent and/or treat bone metastasis, we must gain a better understanding of the molecular mechanisms involved in dissemination of breast cancer to bone.

In order to study the mechanisms involved in breast cancer metastasis to bone, it is important to have animal models that represent the full scope of the metastatic process from the primary tumor to the bone, and that display characteristics similar to human breast cancers. However, developing animal models that fulfill these requirements has proven difficult. This review will discuss the utility and limitations of animal models that are currently being used, as well as new models under development that may prove useful for future research.

2 Transgenic models of metastasis

Historically, overexpression of oncogenes has been the primary method used to study breast cancer in transgenic mice. Expression of the oncogene can be restricted to the mammary gland by using a mammary-specific promoter. One that is widely used is the Mouse Mammary Tumor Virus (MMTV) retroviral promoter and enhancer elements [5]. This promoter has been used to drive the overexpression of several oncogenes originally identified in human breast cancers such as Her2/neu, Ras, and Myc [6–10]. The transgenic mice develop mammary tumors spontaneously, which allows the study of the complete tumorigenic and metastatic processes. A strength of these models is that tumors develop in immune-competent hosts, which allows for the study of interactions between the immune system and the tumor. Unfortunately, bone metastases occur rarely, if at all, in these models and the histopathology of the primary tumors does not fully mimic features of the human disease [11]. The

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MMTV promoter has also been used to drive the expression of the polyomavirus middle T oncogene (PyMT) [12]. PyMT has the ability to transform cells by mimicking a constitutively active membrane receptor and acting as a docking site for tyrosine kinases [13]. This model has been used extensively because it shares many characteristics with human breast tumors. The transition from hyperplasia to the malignant stages is represented and hormone receptors are gradually lost [12, 14, 15]. However, this model has faced criticism due to the fact that PyMT does not itself drive human breast cancers [11]. In addition, this model is also incapable of metastasizing to bone [11, 16].

Despite their pitfalls, transgenic mouse models have been successfully utilized to make important discoveries relevant to tumor growth, invasion, tumor-immune interactions, and the metastatic process (particularly to lungs). While few discoveries in the process of spontaneous bone metastasis have been made using the original MMTV–PyMT mice or other transgenic models, we have shown that overexpression of macrophage stimulating protein (MSP) was sufficient to drive spontaneous metastasis of PyMT-initiated tumor cells from the mammary fat pad to bones in approximately 20 % of mice [16]. These experiments were further supported by examining human breast tumors for activation of the MSP pathway, which revealed a significant correlation between MSP pathway activity and shorter survival times due to increased metastasis to bones and other organs. These findings suggest that even poorly metastatic tumors arising in transgenic mouse models can be tailored to identify and validate modifier genes that are important for bone metastasis.

3 Experimental metastasis models

Due to the apparent lack of bone metastasis in most spontaneous tumor models, alternative models have been developed in an attempt to study this specific metastatic process. Such models often involve transplantation of tumor cells into sites other than the orthotopic tumor site and, therefore, often do not represent the complete metastatic process. Hence, these models are termed "experimental metastasis" models. One example is the intracardiac injection model, which involves direct injection of tumor cells into the left ventricle of the heart. This model is often used to study the metastatic behavior of human breast cancer cell lines in immune-compromised hosts. Intracardiac injections allow broader dissemination of tumor cells to various organs, but skip key steps in the metastatic process such as cell–cell detachment, invasion of the local tissue, and intravasation. However, this model has been used successfully to study the ability of tumor cells to seed and colonize bones, and to develop bone-tropic sub-lines of the MDA-MB-231 human breast cancer cell line. These sub-lines have been analyzed in detail to identify and study genes that are involved in the ability of tumor cells to survive in the bone microenvironment [17, 18]. These genes include FGF5 and CTGF, two factors that stimulate angiogenesis; IL-11, an activator of osteoclast differentiation; MMP1, a bone matrix-degrading metalloproteinase; and CXCR4, a bone-seeking chemokine receptor [17]. Experimental metastasis assays were also successfully used to identify clinically relevant microRNAs that inhibit metastasis to bone and lungs [19]. Identification of genes that specifically influence bone metastasis versus metastasis to other organs [17, 20, 21] argues that the tumor microenvironment of bone metastasis heavily influences the behavior of tumor cells (see below).

A second "experimental metastasis" model involves injecting tumor cells via an intravenous route, which facilitates embolism of tumor cells in the lung capillary vessels. This is a common method used to study lung metastasis, although certain cancer cell lines are able to escape the lungs and go on to form bone or liver metastases [22].

The final "experimental metastasis" model frequently used to study bone metastasis involves injection of tumor cells directly into a bone marrow cavity, commonly the tibia. While this model also does not recapitulate many of the early steps in the metastatic process, it can be used to investigate the ability of tumor cells to colonize the bone, as well as the complex interactions that occur between the tumor and the bone microenvironment. In fact, this model was used successfully to first establish a key set of interactions between tumors and bone, termed the "vicious cycle" [23]. The vicious cycle describes the mutually beneficial interactions that occur between tumor cells and the bone resorbing cells, osteoclasts. When osteoclasts resorb the bone matrix, various growth factors are released into the microenvironment from storage in the bone. A key example is TGFβ, which is then capable of influencing tumor cell survival and proliferation. TGFβ also causes tumor cells to produce the factors IL-11 and parathyroid hormone-related protein, which lead to further osteoclast differentiation [20]. This set of interactions leads to a "vicious cycle" of bone resorbtion and tumor cell growth. Besides revealing key tumor–bone interactions, the experimental metastasis models have also proven useful in testing agents that block osteoclast activity or differentiation, such as bisphosphonates and Denosumab, respectively [24, 25].

4 Orthotopic tumor transplant models

Orthotopic injection of tumor cells or cell lines into the mammary fat pad of mice is also used to study metastasis. Unlike the experimental metastasis models, orthotopic models require the tumor cells to undergo the full spectrum of the metastatic process: formation of primary tumors, cell–cell detachment, local tissue invasion, extravasation,

seeding, and colonization [26]. Because this type of model is likely to more closely replicate the steps required for a tumor cell to metastasize, it might be preferable to experimental metastasis models. Unfortunately, most human breast cancer cell lines are, for some unknown reason, incapable of metastasizing to bone when transplanted orthotopically. However, the orthotopic method has proven particularly successful with a sub-line of the 4T1 mouse mammary tumor cell line, 4T1.2 [27]. The 4T1 cell line was originally isolated from a spontaneous mammary tumor in a BALB/c mouse [28]. When injected orthotopically, 4T1 tumors are capable of metastasizing to bone, albeit infrequently. Bone metastases were then isolated and developed into a sub-line that is highly metastatic and displays tropism to bone (4T1.2) [27]. Because the cell lines can be transplanted into immune-competent syngeneic hosts, the influence of the immune system and other tumor–stroma interactions on the metastatic process can be evaluated. The 4T1.2 model has also been used to demonstrate important roles of T lymphocytes, dendritic cells and macrophages in tumorigenesis and metastasis [29].

5 Humanized mouse models and human tumor grafts

In an attempt to more closely recapitulate human breast cancer and the human tumor microenvironment, various models have been developed which involve "humanized" sites within the mouse. In one model, the mouse mammary fat pad was "humanized" by introducing immortalized human breast fibroblasts into cleared mouse fat pads. Human breast epithelial and stromal cells were then engrafted, creating a more human-like environment for human xenografts [30]. This model allows for the development of human breast ducts and lobules as well as the engraftment of primary human breast cancer cells [30]. Whether this model leads to an increase in spontaneous bone metastasis has not been reported to date. Another model provides a human-like site of metastasis by implanting human bone tissue or bone-like matrix in the flank of an immune-deficient mouse, in conjunction with transplantation of human breast cancer cell lines into the mammary fat pad. In the case of the implanted human bone fragments, one breast cancer cell line was able to form bone metastases and did so only to the human bone implant, not to mouse bones [31]. Use of a similar system with engineered bone scaffolds may allow increased environmental control depending on the cell types that are incorporated into the scaffold [32]. While these models necessarily lack an intact immune system, they suggest that creating a more human-like environment, either at the primary site or the metastatic site, may better facilitate human breast cancer bone metastasis in a mouse model. So far, these models have only been tested using breast cancer cell lines; however, use of primary human breast cancers may better recapitulate the biology of the tumors. We have successfully established passageable tumor grafts derived directly from breast cancer patients. These orthotopic tumor grafts recapitulate the patients' tumors as assessed by histopathology, molecular marker expression, hormone dependence, gene expression, and DNA copy number. Most importantly, the tumor grafts displayed similar sites of spontaneous metastasis as seen in the patient, including at least one line that displayed spontaneous bone metastasis [33]. While only a few reports have been published utilizing these new mouse models as of yet, it is likely that they will provide important new insights into the molecular mechanisms involved in breast cancer metastasis.

6 Techniques for imaging bone metastases

With the development of any animal model of metastasis, the ability to visualize the metastases *in vivo* must be a key component. Imaging bone metastases allows for the study of tumor progression, metastasis, and response to therapeutics. Several methods for imaging bone metastases have been developed which allow for sensitive, non-invasive visualization of tumor development. Several of these small animal imaging technologies replicate the techniques used for human imaging such as micro-computed tomography (CT), micro-positron emission tomography, X-ray and magnetic resonance imaging. However, there are also techniques without a human correlate such as optical imaging of bioluminescence and fluorescence imaging which have proven very useful.

X-ray is a common form of imaging performed to visualize the presence of bone metastases by indirectly viewing the destruction they cause. X-ray imaging is easy to perform and can be done over a time course of an experiment. However, X-rays are only useful if the bone metastasis exerts some morphological change on the bone: for example, bone loss due to an osteolytic metastasis. This imaging technique therefore provides little information in terms of tumor growth, molecular interactions, or early events in the metastatic process such as colonization, due to the time it takes for bone destruction to be visible to X-ray.

Micro-CT has been developed for use on small animals and provides a significant improvement in sensitivity over conventional X-ray. Micro-CT allows for much more accurate and varied quantification of bone destruction due to bone metastases as well as improved image resolution. However, because micro-CT also mainly detects bone destruction and not the tumor itself, it still carries the same drawbacks as X-rays. In addition, micro-CT is not as widely available and can be costly.

Optical imaging has the potential to allow for semi-quantitative measurement of tumor progression, metastasis

and treatment response. In addition, molecular imaging probes can detect the physiological or biochemical interactions that are involved, they are not limited to detection of structural changes. Green fluorescent protein (GFP) has been used extensively and is successful when used to label specific cell types, either tumor cells or cells in the host. While GFP is still used as a marker for imaging, it has the drawbacks of interfering auto-fluorescence and absorption of the signal by tissues. Because of these drawbacks, use of red fluorescent protein and the newer near-infrared fluorescent (NIRF) probes are becoming popular. These probes have significantly less auto-fluorescence and allow more penetration due to a decrease in the tissue absorbance. New markers are being developed which allow one to monitor the activity of a specific protein or pathway, improving the understanding of the molecular pathways involved in bone metastasis. One benefit of using specific NIRF probes is that they do not require the modification of cells with reporter constructs. An example of this is a quenched probe which becomes fluorescent as it undergoes proteolytic cleavage by matrix metalloproteinases [34].

Bioluminescence utilizing various luciferase genes is extensively used in animal models of bone metastasis. This technology allows for the quantification of tumor growth, sensitive detection of the bone micrometastases, and labeling of a specific gene or pathway through gene reporters [35]. An added benefit of this method is that it allows for the direct visualization of an inhibitor of a pathway, functioning in real time. Recently, dual bioluminescence imaging was used to dissect the functional dynamics of the TGFβ pathways in breast cancer metastasis [36, 37]. The use of imaging reporters allowed detailed analysis of TGFβ activity with inducible constructs and in the presence of pharmacological inhibitors. This technique allowed the authors to monitor the functional importance of this key pathway during different stages of metastasis.

Molecular imaging will evolve as new information into the complex interactions between tumor cells and the microenvironment is acquired. Because these imaging techniques allow visualization of biological processes as they occur, they will be of great importance for any *in vivo* model of bone metastasis.

7 Conclusions

Breast cancer bone metastasis significantly impacts quality of life for patients and represents a serious public health problem. In order to develop new therapies that prevent and/or treat bone metastasis, we must gain a better understanding of the molecular mechanisms involved in the dissemination of breast cancer to bone. One hindrance to new discoveries is the relative lack of animal models that display the full scope of the metastatic process and that display characteristics similar to human breast cancers. The development of more mouse models that faithfully recapitulate metastasis, as well as the advent of significant technical advances in metastasis imaging, should lead to a new understanding of pathways involved in bone metastasis and identification of mechanisms to prevent or treat metastasis of breast cancer to bone.

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CHAPTER 3

THE MACROPHAGE STIMULATING PROTEIN/RON PATHWAY AS A POTENTIAL THERAPEUTIC TARGET TO IMPEDE MULTIPLE MECHANISMS INVOLVED IN BREAST CANCER PROGRSSION

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The Macrophage Stimulating Protein/Ron Pathway as a Potential Therapeutic Target to Impede Multiple Mechanisms Involved in Breast Cancer Progression

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Abstract: Macrophage Stimulating Protein (MSP) is the only known ligand for the receptor tyrosine kinase Ron. The MSP/Ron pathway is involved in several important biological processes, including macrophage activity, wound healing, and epithelial cell behavior. A role for MSP/Ron in breast cancer has recently been elucidated, wherein this pathway regulates tumor growth, angiogenesis, and metastasis. Here, we review the recent literature surrounding MSP/Ron function in tumor cells, inflammatory cells, and osteoclasts – cell types that often coexist in breast tumor microenvironments. We discuss the potential implications of MSP/Ron activity occurring concurrently in these cell types on tumor progression and metastasis. Lastly, we outline the potential for targeting MSP/Ron as a novel therapy for breast cancer, and for other cancer types.

Keywords: Breast cancer, macrophage stimulating protein, metastasis, MSP, MST1R, osteolysis, Ron, therapeutic target.

INTRODUCTION

Breast cancer has a relatively low case-fatality rate, but approximately 20% of women diagnosed with breast cancer eventually develop metastatic disease. Because breast cancer is highly prevalent and metastatic breast cancer is rarely curable, a significant number of women, about 40,600 in the U.S. per year, will die of the disease [1]. Although the incidence of breast cancer is much higher in women over 50, breast cancer is the major cause of death from all reasons in women age 35-50 and represents a major health care and societal problem.

The goal of initial treatment is to reduce the risk of both local and systemic recurrence. Initial treatment for localized breast cancer is designed to reduce the risk of in-breast and regional disease; local recurrence in breast, skin, subcutaneous tissues and axilla rarely causes death but often results in significant morbidity and can give rise to systemic metastases [2]. Local therapy begins with surgery: either lumpectomy to remove the tumor from the intact breast, or mastectomy. Radiation therapy reduces the risk of local recurrence and is almost always recommended after lumpectomy. Radiation is also generally recommended after mastectomy if the tumor is large or involves regional lymph nodes. Initial treatment of breast cancer often also includes systemic therapy designed to eradicate occult metastatic disease that is not clinically evident, but which may eventually cause relapse and death. Treatment given in this setting is termed "adjuvant therapy" and, because of the near-universal fatality of metastatic breast cancer, is recommended for the majority of women newly diagnosed with breast cancer in order to improve disease-free survival [3].

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Systemic therapy for breast cancer may be given either as adjuvant therapy or as treatment of metastatic disease, and includes hormonal therapy, chemotherapy, and/or biological agents. Hormonal therapy is used if the malignant cells express estrogen and/or progesterone receptors. Chemotherapy of several different classes can be effective in all types of breast cancer, independent of hormone receptor expression. Targeted therapies used in breast cancer include trastuzumab, a monoclonal antibody approved for use in combination with chemotherapy for HER2 positive breast cancer, and lapatinib, a small molecule used for HER2 positive metastatic breast cancer. Bevacizumab, a VEGF inhibitor, is also approved for use in metastatic breast cancer [3]. Numerous other agents are being evaluated as potentially effective adjuvant therapies, notably bisphosphonates, which are best known for blockade of osteoclast function. Various bisphosphonates are in late-phase clinical trials and may reduce the risk not only of skeletal metastases, but visceral metastases as well [4].

Patients with metastatic breast cancer have a median survival of approximately two years [5]. Women with hormone-sensitive metastases limited to bone and soft tissue have on average a longer survival, while those with extensive parenchymal organ involvement usually have a shorter survival, particularly if the tumor does not express hormone receptors or HER2. These latter tumors are aggressive, and there are no targeted therapies available for this subtype of breast cancer. Clearly, there is a need for new therapies with greater efficacy against this disease, and a particular need for therapies that might reduce or prevent the growth of metastatic lesions. Targeted therapies are generally less toxic than other anti-cancer agents and, when effective, are invaluable in achieving the goal of treatment of metastatic breast cancer: to achieve disease control while avoiding toxicity due to therapy. Here, we discuss the exciting potential for a new therapeutic target in breast cancer: the receptor tyrosine kinase Ron.

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THE MET/RON FAMILY OF RECEPTOR TYROSINE KINASES

The receptor tyrosine kinase Ron (also known as human MST1R, for macrophage stimulating 1 receptor, and as murine Stk1, for stem cell kinase 1) is the cell surface receptor for macrophage stimulating protein (MSP; also known as MST1, for macrophage stimulating 1, and as HGFL, for hepatocyte growth factor-like). In humans, Ron is one of only two members of a distinct receptor tyrosine kinase (RTK) family that also includes Met. The highest amino acid identity between Ron and Met is located within the kinase domain (63% identity); the other regions are not highly conserved (34% overall). The respective ligands for Ron and Met are also similar; MSP is 45% identical to hepatocyte growth factor (HGF), the Met ligand. Both ligands are glycoproteins that are secreted as inactive single-chain peptides and are proteolytically processed into active, disulfide-linked α/β heterodimers [6]. HGF binds and activates Met and MSP binds and activates Ron [7-9]; although there is crosstalk between Ron and Met intracellular signaling [10, 11] the ligands and receptors are not interchangeable [12].

Like their ligands, Met and Ron are cleaved disulfide-linked heterodimers. The mature receptors consist of extracellular α and β chains, involved in ligand binding, and the intracellular portion of the β chain, which is responsible for signaling. Binding of ligand causes receptor homodimerization and phosphorylation of two tyrosine residues within the catalytic site, which regulates kinase activity [8, 13]. Activation of kinase activity results in phosphorylation of the carboxy-terminal docking site of the receptor. The docking site is essential for downstream signaling through direct and indirect binding of SH2 domain-containing adaptor proteins such as Grb2, PI3K, and Src [14]. Ron and Met are both expressed in a variety of tissues during development and, in adults, are expressed mainly on epithelial cells and in the nervous system. However, Ron is also highly expressed on adult macrophages and osteoclasts.

Although the signaling pathways that are activated by Ron and Met are similar, they culminate in related, yet distinct, cellular functions. Both are known to induce "scattering," a phenomenon in which cells detach from one another and migrate away from the central colony [10, 15, 16]; both promote proliferation through the MAPK pathway and survival through both MAPK and PI3K/AKT pathways [17-19]; and both have the ability to promote an epithelial-mesenchymal transition, albeit in distinct situations [20, 21]. However, a major effect of MSP/Ron signaling is on the motility and activation of macrophages – a function clearly divergent from that of Met.

Terminally differentiated macrophages express Ron and were first noted to respond to MSP by rapidly altering their shape and increasing chemotactic and phagocytic ability [22, 23]. More recently, it has been realized that MSP/Ron also plays a critical role in attenuation of the inflammatory response. Mice lacking Ron activity display defects in the inflammatory process, most notably the inability to downregulate TNFα and nitric oxide production in response to infection or injury [24-27]. Thus, MSP/Ron signaling plays a dual role in regulating inflammation: initial stimulation of chemotaxis and phagocytosis – important features of "classical" macrophage activation - and, more critically, resolution of the inflammatory response by promotion of the "alternatively activated" macrophage state (discussed in more detail below).

MSP belongs to a group of kringle domain-containing proteins that diverged from an ancient family of serine proteases involved in blood coagulation and fibrinolysis [28]. Amino acid substitutions in the catalytic domain during evolution rendered MSP inactive as a protease, although it retained the feature of being cleaved and activated by other serine proteases. Such cleavage is, in fact, required for the conversion of pro-MSP to the mature, active form of MSP that can bind and activate Ron.

Activation of pro-MSP was originally discovered in wound exudates, where it resulted in stimulation of macrophage activity [23]. A serine protease responsible for activating pro-MSP was localized to macrophage membranes [29] and later identified as matriptase [30]. However, other proteases, such as hepatocyte growth factor activator, also appear to cleave and activate pro-MSP *in vivo* [31]. Pro-MSP is predominantly secreted from the liver, and exists in the blood plasma in its biologically inactive form at a concentration of about 5nM, and is thus poised to initiate Ron signaling upon cleavage [23].

Functional consequences of MSP/Ron signaling are not limited to macrophages or the inflammatory process. Ron, like Met, is upregulated in many types of epithelial cancer, and they are occasionally co-upregulated [32, 33]. Although the role of Met in cancer has been investigated for more than 20 years, culminating in development of multiple targeted therapies now in clinical trials (for a recent review, see [34]), Ron has more recently been recognized as a major player in progression of human epithelial cancers. This report focuses on the significance of the MSP/Ron pathway in breast cancer, and the ensuing opportunities for therapeutic intervention.

RON EXPRESSION AND FUNCTION IN BREAST CANCER

Ron is expressed at very low levels in normal human breast epithelium, but becomes overexpressed in a large proportion of breast tumors (Table **1**). Interestingly, Ron mutation is not associated with breast cancer, suggesting that overexpression of the wild type protein is sufficient to contribute to tumor development or progression. The reason for overexpression has not yet been established. Both MSP and Ron are located on chromosome 3p21.31, and the 3p21 region is often altered in cancer. Specifically, 3p21 undergoes both loss of heterozygosity [35] and amplification in various tumors and cancer cell lines [36, 37]. This region was amplified in 15-42% of lung, renal, and breast cancers examined [36], which suggests that amplification could contribute to MSP and/or Ron overexpression in breast cancer. Consistent with this, MSP is also overexpressed by up to ~20% of early stage human breast tumors [38].

Mouse models have been instrumental for elucidating the contribution of Ron signaling to breast cancer. When overexpressed under the mouse mammary tumor virus (MMTV) promoter, Ron caused mammary hyperplasia by 12 weeks of age, followed by development of adenocarcinoma

in 100% of female mice [39]. Tumors initiated by Ron exhibited spontaneous metastasis to liver and/or lung in ~90% of animals, which is remarkable given the relatively limited metastatic potential of other transgenic mouse models of breast cancer [40]. Confirmation that the Ron pathway is a significant contributor to breast tumor metastasis was obtained by examining 457 breast cancers from two independent patient cohorts [38]. In these studies, co-overexpression of MSP, its activating enzyme matriptase, and Ron (collectively referred to as MSP/matriptase/Ron) was used as a surrogate indicator of Ron signaling activity, and was a significant independent prognostic factor for metastasis and reduced survival. Importantly, overexpression of MSP or Ron mRNA alone did not significantly correlate with patient outcome, suggesting that Ron function in metastasis of breast cancer could be largely ligand-dependent, even when the receptor is overexpressed. Indeed, activation of Ron by overexpression of MSP in a mouse model of mammary cancer (transgenic mice expressing the polyomavirus middle T antigen under the mouse mammary tumor virus promoter; MMTV-PyMT [41]) was sufficient to cause spontaneous metastasis to lung, lymphatics, and bone. Patients whose tumors expressed MSP/matriptase/Ron also exhibited significantly more metastasis to lung, liver, brain, and bone (bone was the most frequent site of metastasis). Furthermore, MSP-induced bone metastases in the mice were osteolytic, as they are in human breast cancer patients, and appear to be the first example of spontaneous metastasis of a primary (non cell line-derived) tumor from the mammary gland to the bone in mice [38]. Thus, MSP/Ron activity exerts a gain-of-function effect in breast cancer, promoting tumor metastasis to clinically relevant sites.

OPPORTUNITIES FOR RON INHIBITOR THERAPY IN BREAST CANCER

Consistent with the knowledge that breast cancer is a complex and remarkably heterogenous disease, single agent targeted therapy has generally not been effective long term, even in combination with standard chemotherapy. This is particularly true for metastatic breast cancer, which is still considered incurable [42]. Our ever-increasing understanding of mechanisms involved in tumor progression suggests that the ability to simultaneously abrogate several independent processes that are critical for cancer progression would hold great promise for new therapeutic approaches.

It is now realized that many of the processes that contribute to tumor progression and metastasis are not actually intrinsic to the tumor cells. Rather, the tumor microenvironment plays a key role in critical processes such as angiogenesis [43], growth factor production [44], tumor inflammation and immunoediting [45], invasion and intravasation [46], as well as modifying the metastatic site to create a hospitable environment [47]. In fact, it has become clear that tumor progression and metastasis in mouse models can be severely restricted or even eliminated by limiting tumor inflammation [48, 49].

Data obtained *in vivo* using sophisticated mouse models and primary human breast cancer specimens strongly suggest that the Ron pathway is an exciting new target for therapy against solid tumors. MSP/Ron not only plays a causal role in tumor development and progression, but also plays a critical role in the type of inflammation that is known to occur in tumor microenvironments [50, 51]; this is discussed in detail below. Based on the known function of Ron in tumor cells, macrophages and osteoclasts, we suggest that Ron inhibition would simultaneously block essential processes both *intrinsic* and *extrinsic* to the tumor cells: tumor growth and angiogenesis, promotion of metastasis by 'alternatively activated' macrophages, promotion of the wound healing process, and osteolysis due to breast cancer bone metastasis. The specific functions of Ron in each of these processes in the 'normal' state are discussed in detail below, followed by a discussion of the implications for Ron activity in the setting of cancer.

TUMOR-INTRINSIC ACTIVITIES: MSP/RON IN TUMOR GROWTH AND ANGIOGENESIS

Investigation of Ron activity in epithelial cancer cell lines has revealed roles in cell proliferation, survival, migration, and epithelial-mesenchymal transition [15, 52-54]. Selective Ron inhibitors have been generated and were reported to affect these processes, indicating that blockade of Ron function is achievable at least in certain settings [18, 55].

As described above, gain of function studies in mouse models have shown that activation of Ron through either overexpression of MSP [38] or overexpression of Ron [39] was sufficient to increase tumor growth as well as both the frequency and tissue tropism of metastasis in mice, and overexpression of MSP/matriptase/Ron significantly correlated with increased metastasis and death in breast cancer patients [38].

Conversely, loss of Ron function has been demonstrated to affect tumor growth, angiogenesis, and metastasis in a mouse model of breast cancer. Mice lacking the tyrosine kinase domain of Ron (Ron TK-/- [56]) were crossed to MMTV-PyMT mice, which resulted in decreased mammary tumor growth and reduced metastasis to lung – the only site of metastasis in the MMTV-PyMT model. This effect occurred in parallel with decreased vasculature and increased apoptosis in the tumors [57]. Selective Ron inhibitors have also shown some efficacy in xenograft models for other types of cancer (see below). Together, these data suggest that abrogation of Ron activity can impair tumor growth and reduce the likelihood of metastasis, and that pre-clinical studies using Ron inhibitors have shown promising results.

TUMOR-EXTRINSIC ACTIVITIES: MSP/RON IN MACROPHAGE ACTIVITY

Ron is expressed on terminally differentiated resident macrophages, but not on mononuclear phagocytes or circulating monocytes; Ron is upregulated during macrophage differentiation [58]. Ron is expressed on many different types of resident macrophages including alveolar macrophages, microglia, peritoneal macrophages, and dermal macrophages from either normal or wounded skin [23, 59, 60].

As suggested by its name, MSP does function to stimulate macrophages. Activated MSP increases the ability of

macrophages to undergo chemotaxis; stimulation of Ron by MSP leads to rapid changes in cell shape and motility [30, 61]. MSP/Ron activity also promotes rapid phagocytosis of C3bi coated erythrocytes *via* complement receptor 3 [62].

Consistent with its function in macrophage stimulation, *in vivo* experiments demonstrate that the Ron pathway is important in protection against Gram-positive bacteria. When Ron -/- mice were challenged with *Listeria monocytogenes* they showed increased bacterial burden and increased susceptibility to the infection - a phenotype similar to that of interferon-gamma (IFNγ) knockout mice and tumor necrosis factor-alpha (TNF-α) knockout mice. Lack of Ron function may manifest itself in the inability of macrophages to efficiently eliminate the bacteria, as rapid clearance by macrophages *via* the complement receptor is known to be essential in preventing *Listeria* infections [62].

Studies of MSP/Ron signaling in macrophages indicate that, although the Ron pathway is involved in macrophage activation and protection from particular microorganisms, it is *critical* for resolution of inflammation in many models. Mice lacking Ron activity are viable and fertile but have noteworthy defects in macrophage function; Ron is necessary to limit inflammatory responses [56, 63]. Peritoneal macrophages isolated from Ron deficient mice produce increased levels of nitric oxide in response to lipopolysaccharide (LPS) stimulation, and when Ron TK-/- mice are challenged with sub-lethal doses of LPS, they are more susceptible to LPS-induced endotoxic shock [56].

The mechanisms by which MSP/Ron signaling functions to resolve inflammation are elegantly studied. One important function of Ron is to downregulate interleukin 12 (IL-12) production in macrophages. The inability of Ron TK-/- mice to downregulate IL-12 leads to increased IFN-γ production by natural killer cells, and a prolonged inflammatory reaction [50]. *In vitro*, MSP is sufficient to polarize macrophages from the "classically activated" to the "alternatively activated" state [64] (also known as the M1 and M2 states, respectively, and further described below). MSP/RON signaling also suppresses inflammation through several other routes: activation of suppressors of cytokine signaling, down-regulation of IFN-γ, reduction of major histocompatability complex class II cell surface expression, and reduction of IFN-γ-induced STAT1 phosphorylation [50]; downregulation of inducible nitric oxide synthase (iNOS) [64]; increased production of the anti-inflammatory cytokine IL-10 [65]; and downregulation of cyclooxygenase-2 (COX-2) expression through inactivation of Nuclear Factor kappa B (NFkB) [66].

Taken together, the published data indicate that the MSP/Ron pathway plays a dual role in inflammation: a role in initial macrophage activation, as well as an important role in downregulating the inflammatory response. Ron activity results in increased migration of macrophages to sites of infection, and stimulates phagocytosis early in the infection process. Later, Ron is required to resolve inflammation by downregulating iNOS and IL-12 and by upregulating IL-10. Still, much is to be learned: the different roles for Ron signaling in infections elicited by gram-positive versus gram-negative bacteria indicate that MSP/Ron function is context dependent, and the interaction of MSP-stimulated macrophages with cells of the acquired immune system is yet to be discovered.

AN INTERSECTION OF INSTRINSIC AND EXTRINSIC FACTORS: MSP/RON IN WOUND HEALING

Skin wound repair is essential for tissue homeostasis and involves three phases: inflammation, proliferation, and remodeling (for review see [67]). Inflammatory cells play a crucial role in the wound healing process. Macrophages remove dead tissue, stimulate the growth of new blood vessels, regulate fibroblast recruitment, re-growth of the epithelium, and remodeling of connective tissue (for review see [68]). Classically activated macrophages (M1 macrophages) are present early during the wound healing process and function to remove pathogens and stimulate the immune response, whereas alternatively activated (M2) macrophages predominate later in the repair process. M2 macrophages fail to present antigen to T cells, produce minimal amounts of pro-inflammatory cytokines and nitric oxide, and are less efficient than M1 macrophages at killing microbes. Instead, M2 macrophages secrete extracellular matrix (ECM) proteins and polyamines, which influence production of cytokines, inhibit clonal expansion of lymphocytes, and stimulate proliferation of epithelial cells [69]. M2 macrophages are characterized by downregulation of iNOS and upregulation of arginase 1, which metabolizes arginine to urea and ornithine. Consequently, there is increased arginase activity in experimental rat wounds, along with increased ornithine levels [70]. Importantly, MSP/Ron signaling is instrumental in the switch from expression of iNOS to expression of arginase [64].

Successful wound repair entails resolution of the inflammatory response and, as discussed above, MSP is both necessary and sufficient to induce M2 macrophage polarization, which assists in attenuation of inflammation. MSP/RON signaling is involved at various steps of the wound healing process. In experimental excisional wounds in rats, immunostaining revealed both MSP and Ron within the wound, where maximum staining occurred between 7 and 21 days post-wounding [71]. There are also increased levels of activated MSP in fluids collected from burn wounds in humans, and RON-expressing macrophages are scattered throughout the dermis.

In addition to resolving inflammation in wounds, Ron plays a role in repairing wounded skin. This process, referred to as re-epithilialization, involves migration and proliferation of epidermal keratinocytes. Cells at the wound margin loosen their extracellular matrix (ECM)-cell and cell-cell interactions in order to migrate across the wound (for review, see [72]. Ron is upregulated by proliferating and differentiated populations of keratinocytes [23], and MSP promotes keratinocyte migration in mouse wounds and in wound healing assays *in vitro*. In primary keratinocytes, the 14-3-3 protein associates with Ron in response to MSP signaling, which induces spreading and improved migration on laminin 5 ECM [73]. It is notable, however, that MSP deficient mice do not show any defects in specific skin wound healing models [74], suggesting that functional redundancies exist for this important biological process.

The role of MSP/Ron in other models of injury has also been investigated. In two different models of lung injury, Ron proved to be essential for protection from unregulated inflammation. When injected with intrapulmonary LPS, mice lacking Ron function display increased lung injury and damage due to overproduction of nitric oxide and TNFα through the NFkB pathway. Again, MSP/Ron function was deemed necessary to suppress NFkB activation *in vivo* [75]. In a nickel-induced acute lung injury model, in which mice are exposed to aerosolized nickel particles, mice lacking RON function exhibited significantly decreased survival times compared to control mice. The mice showed increased levels of IL-6 and the chemokines Chemokine (C-C motif) ligand 2 (CCL2) and Chemokine (C-X-C motif) ligand 12 (CXCL12), as well as increased serum nitrite levels. These effects were commensurate with earlier onset of pulmonary inflammation, edema and lethality [76]. Gene expression analysis indicated that genes responsible for inflammation, edema and lymphocyte function were significantly altered in mice lacking Ron activity [77].

Paradoxically, in a model of LPS-induced acute liver failure in galactosamine-sensitized mice, RON deficient mice are actually protected from liver injury. This finding was based on histological analysis as well as serum alanine amino transferase levels, and was associated with decreased number of liver cells undergoing apoptosis [78].

The last three examples of Ron involvement in injury indicate that the cytokine milieu and the type of injury likely influence the outcome of MSP/Ron signaling. Data from mice lacking Ron activity indicate that blocking MSP/Ron signaling therapeutically may not adversely affect healing of common skin wounds, but could be a concern for life-threatening infections. An important consideration, however, is whether acute loss of Ron function (as would occur with therapeutic blockade of Ron signaling) would have different effects than the chronic lack of function that develops in genetically engineered mice.

INFLAMMATION AND CANCER: DUAL FUNCTION OF MSP/RON?

A growing body of evidence supports the idea that inflammation contributes to cancer development and progression (for review, see [45]). The risk of developing cancers of the esophagus, colon, pancreas, lung, and gallbladder is heightened by the presence of chronic inflammatory diseases. Chronic inflammation, like chronically unhealed wounds, is characterized by a prolonged cycle of tissue damage, cellular proliferation and tissue repair [79]. The inflammatory environment is enriched with macrophages that generate high levels of reactive oxygen and nitrogen species to fight infection. However, when unregulated, these agents can react with DNA and cause mutations in proliferating epithelial and stromal cells (for review, see [80]).

Increased tumor associated macrophage (TAM) density is associated with tumor progression and metastasis (for review, see [81]). TAMs have many characteristics of an M2 activation phenotype, and are thought to contribute to tumor development by releasing IL-10 and PGE2, which suppress the inflammatory reaction to the tumor [82]. TAMs also release pro-angiogenic factors such as vascular-endothelial growth factor (VEGF), endothelin 2 and plasminogen activator, and pro-proliferative factors such as epidermal growth factor (EGF), fibroblast growth factor, HGF, platelet-derived growth factor, transforming growth factor β (TGFβ), and IL-6 [83, 84]. TAMs are also thought to facilitate tumor cell invasion and metastasis by releasing MMP2 and MMP9, which modify the ECM and basement membrane, and by facilitating a paracrine loop of EGF and colony stimulating factor-1 signaling to promote metastasis [85]. Thus, TAMs endow tumors with an environment that enhances the survival, migration and proliferation of epithelial cells and are a large contributor to the observation that tumors are much like chronically unhealed wounds [86].

Although there is insufficient evidence at this time to suggest that MSP/Ron-induced inflammation *directly* participates in cancer progression, studies indicate that MSP is able to evoke dose-dependent superoxide anion production in human alveolar macrophages *via* src, MAPK, and p38 signaling pathways [59]. In human alveolar macrophages from either smokers or non-smokers, MSP efficiently activates NF-kB. However, MSP evokes superoxide production, cytokine release and NFkB activation to significantly higher levels in cells from smokers versus those from non-smokers, indicating that MSP may enhance inflammation due to cigarette smoke [65]. Although this may contribute to tumorigenesis, there is another likely, and potentially more impactful, role for MSP/Ron in tumor progression and metastasis: polarization of TAMs to an M2 phenotype.

CONSEQUENCES OF MSP/RON ACTIVATION IN TUMORS

As described above, pro-MSP is present in high concentrations in serum, and conversion of pro-MSP into MSP occurs locally at sites of inflammation [23]. A serine protease that was shown to cleave and activate MSP, matriptase, is normally present on macrophages, but is also upregulated in a large percentage of breast cancers [30]. In addition, RON is overexpressed to high levels (and Ron is phosphorylated) in ~50% of breast cancers [87]. It is reasonable to presume that activation of MSP locally, at sites of inflammation in tumors, would not only lead to activation of Ron on TAMs, but also on the tumor epithelium, where Ron has been shown to induce proliferation, survival, cell migration, EMT, invasion, and metastasis (see above).

Although the result of Ron signaling in TAMs is still unclear, MSP/Ron activates signaling pathways in macrophages that are known to be involved in tumor progression. Ron activation causes phosphorylation of the signal transducer and activator of transcription 3 (STAT3) protein [88], which is required for the immunosuppressive and tumor promoting effects of TAMs. In fact, STAT3 knockout mice [89] show similar inflammatory phenotypes as RON deficient mice, and several infectious agents are known to cause inflammation-induced cancer *via* STAT3 activation [90]. Furthermore, the MSP/Ron-induced cytokine IL-6 activates STAT3 in both inflammatory cells and epithelial cells [90].

In addition to the immunosuppressive effects of MSP/Ron *via* STAT3 activation, the MSP/Ron pathway also

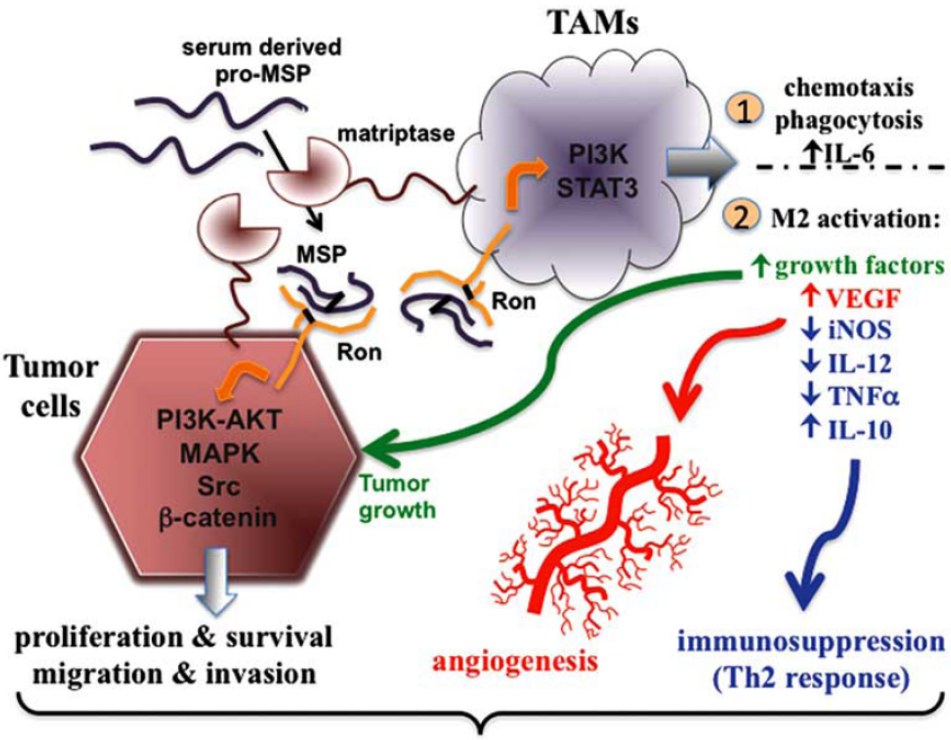

Fig. (1). Model for the contribution of MSP/Ron function in tumor progression and metastasis through both cell autonomous (tumor cell proliferation, survival, migration and invasion) and non-cell autonomous (macrophage activation and polarization) functions.

down-regulates STAT1 activity [27], which is involved in anti-tumor immune responses through upregulation of IL-12 [90]. STAT1 and STAT3 clearly act in opposing roles with regard to immune responses against tumors; genetic deletion of STAT3 in immune cells leads to upregulated STAT1 activity and increased anti-tumor properties [91]. Thus, it is likely that both STAT3 activation and STAT1 inhibition by MSP/Ron may manifest in immune tolerance to the tumor, in addition to the potential function of MSP/Ron in promoting secretion of pro-growth and pro-angiogenic factors by M2-polarized TAMs. The potential consequences for MSP/Ron activation in tumors are summarized in Fig. (**1**).

MSP/RON IN OSTEOLYTIC BONE METASTASIS

In addition to its expression and activity in macrophages, Ron is also expressed on osteoclasts, the specialized macrophages of bone. Ron becomes expressed on the surface of multinucleated osteoclast-like cells when human bone marrow cells are differentiated *in vitro*, and MSP activates osteoclasts, causing bone resorption [92]. *In vivo*, Ron is highly expressed on osteoclasts but does not appear to play a critical role in bone development, since mice lacking Ron function have no overt bone defects [56].

The role of MSP in osteoclast activation is highly relevant to breast cancer, since bone metastasis occurs in 70-80% of patients and is therefore the most common site for relapse [93, 94]. Osteoclasts are activated *in vitro* by breast cancer cells that express MSP, and to a much greater extent than that induced by control tumors [38]. Furthermore, mice with mammary tumors expressing MSP spontaneously developed osteolysic bone metastasis, and breast cancer patients with high MSP/matriptase/Ron experienced significantly more metastasis to bone than those without high MSP/matriptase/Ron [38]. A model for the role of MSP/Ron activity in osteolytic bone metastasis is shown in Fig. (**2**). A specific understanding of whether the MSP/Ron pathway contributes to the "vicious cycle" of breast tumor growth in bone that was previously proposed by Guise and Mundy (for recent reviews on breast cancer bone metastasis, see [93, 95]), or whether MSP/Ron activation defines a new mechanism for osteolysis remains to be determined. Understanding the role of the MSP/Ron pathway in breast cancer bone metastasis would have important clinical implications.

MSP/RON AS A THERAPEUTIC TARGET

The ability to simultaneously block several key pathways that contribute to tumor progression might lead to more efficacious therapy. We suggest that the MSP/Ron pathway holds promising potential in this regard, since it is upregulated in a large proportion of cancers and contributes

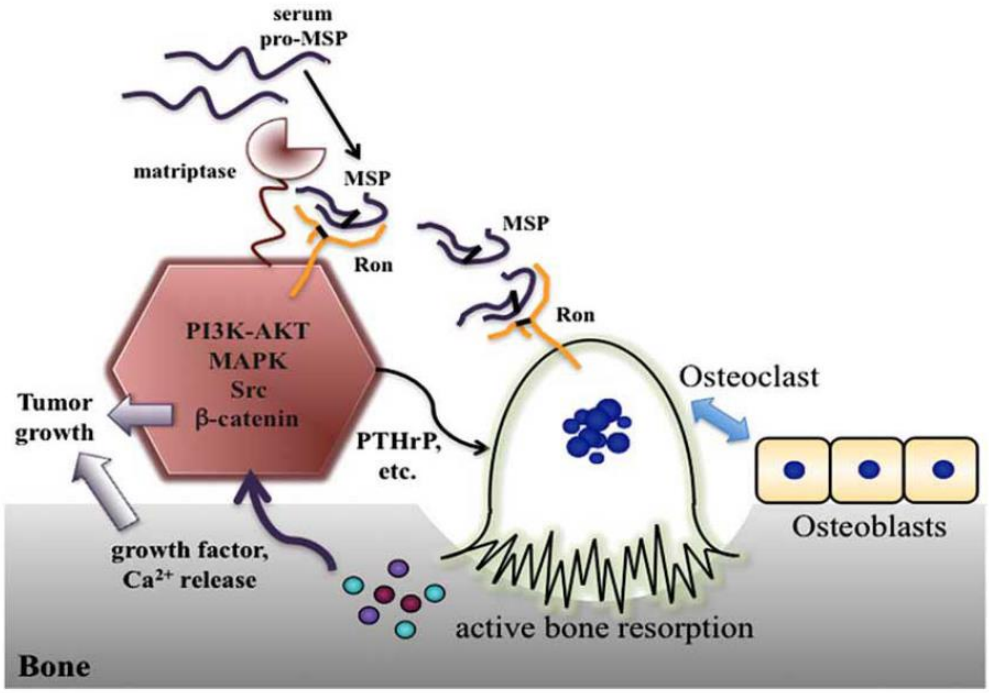

Fig. (2). Model for the role of MSP/Ron activity in osteolytic bone metastasis as a complication of breast cancer. MSP can directly activation osteoclast activity through Ron stimulation. The ensuing bone resorption can release calcium and growth factors that stimulate tumor growth and perpetuate a "vicious cycle" [93].

to proliferation, survival, migration, and invasion of tumor cells. In addition, though, MSP/Ron activity also promotes M2 macrophage polarization, potentially leading to secretion of immunosuppressive cytokines as well as growth and angiogeneic factors that support the tumor. Blockade of MSP/Ron function might therefore interfere with critical tumor-promoting pathways in the tumor itself *and* in the tumor microenvironment.

Strategies for Ron Inhibition

One can imagine several potential strategies to interfere with MSP/Ron function, including prevention of pro-MSP activation, blockade of MSP-Ron interaction and/or receptor dimerization, and inhibition of Ron kinase activity. Inhibition of MSP activating enzymes such as matriptase is unlikely to be effective due to redundancy between several serine proteases capable of activating MSP *in vitro* and *in vivo* [30, 31, 96]. Strategies to prevent ligand-receptor interactions and/or receptor downregulation could be achieved through generation of monoclonal antibodies (mABs). mABs can also have the added benefit of inducing antibody-mediated cellular cytotoxicity, analogous to that achieved by the HER2 antibody trastuzumab (Herceptin) in breast cancer. One Ron inhibitory antibody has been described, and was shown to be effective in slowing growth of colon, lung, and pancreatic cancer xenografts [18].

Inhibition of kinase activity may be less specific, due to high conservation of kinase domains within receptor tyrosine kinases, but would have the added benefit of oral availability and potentially lower cost. One advantage of targeting Ron with a small molecule kinase inhibitor is that Met kinase inhibitors are already available, some of which are being tested in clinical trials [34]. Since the kinase domains of Ron and Met are 68% identical, it is very likely that a Met inhibitor will also block Ron, at least to some extent. A dual Ron/Met inhibitor recently showed promising results in xenograft studies using Met-dependent cell lines or colon cancer cells expressing an endogenous, hyperactive form of Ron (see below) [55].

Challenges for Drug Development Against MSP/Ron

As with all potential new therapies, there are great challenges. The MSP/Ron pathway, in particular, may be even more confounding due to dual effects on the tumor and on the host immune system. One hurdle in drug development for oncology is pre-clinical testing in animal models, and immunodeficient mice are routinely used for initial studies. However, if MSP/Ron functions to promote tumor progression and/or metastasis through alteration of immune function, the results would be very difficult or impossible to discern in such a model. Use of syngenic, immunocompetent mouse models such as the one we developed [38] can overcome the problem of immune involvement, but pre-

cludes testing species-specific drugs such as anti-human Ron antibodies. Likewise, if activation of host macrophages is a key component of MSP/Ron function in tumors, drugs that recognize and inhibit both the human and murine Ron proteins would be required for validation in xenograft models.

The existence of multiple isoforms of Ron poses another challenge for drug development. There are a number of alternative Ron isoforms described. These include hyperactive splice variants [97, 98], and an N-terminally truncated form of Ron, termed short form Ron (sfRon). sfRon is generated through utilization of a second, internal promoter within intron 10 of *RON,* creating a constitutively active form of the receptor that does not require ligand binding for activity [99]. sfRon is expressed in cell lines originating from multiple cancer types, and has been detected in primary breast cancers [99]. In mice, sfRon is required for transformation of erythroblasts by the Friend virus, and mice with a naturally occurring polymorphism in the sfRon promoter are resistant to this form of erythroleukemia [100]. Since the human sfRon promoter is relatively uncharacterized, it is unknown whether polymorphisms exist and are relevant to tumorigenesis. However, methylation of the main RON promoter may contribute to expression of sfRon in cancer cell lines [101]. Upregulation of active forms of Ron in cancer could serve as an important contributor toward resistance to therapies designed to interfere with ligand binding, for example. Thus, in our view, Ron kinase inhibitors may hold the greatest promise for targeted therapy against this pathway in breast cancer.

RELEVANCE OF THE MSP/RON PATHWAY IN OTHER CANCERS

The Ron pathway may also be an excellent therapeutic target in cancers other than breast. Ron is overexpressed in a wide variety of human cancer tissues (Table **1**) and, although its function in the epithelial compartment is not understood for all of these malignancies, the function of MSP/Ron in tumor inflammation is likely to be conserved. MSP and matriptase are also upregulated in many cancers [30, 38, 102, 103], further supporting the idea that both autocrine and paracrine pathways could contribute to tumorigenesis and/or progression of malignancy.

Ron is overexpressed in small cell lung carcinoma (SCLC) cell lines, a pulmonary carcinoid cell line, and in non-small cell lung carcinoma (NSCLC) [102, 103]. While MSP expression is low to undetectable in both SCLC and pulmonary carcinomas, MSP is expressed in NSCLC primary tumors and cell lines [103, 104]. Addition of MSP to NSCLC cell lines expressing Ron resulted in increased cell motility [103]. In addition, overexpression of Ron in distal lung epithelial cells results in the development of lung adenomas *in vivo* [105].

Expression of full length Ron, as well as various isoforms of Ron, has been demonstrated in human colon cancer cell lines as well as primary adenocarcinomas [97, 106, 107]. Ron is highly expressed in 60% of colorectal adenocarcinomas and its expression correlates with the degree of differentiation of these tissues [106, 107]. The constitutively active splice variants RONΔ155, RONΔ160, and RONΔ165 are most notably expressed in colon cancers. Expression of RONΔ155 or RONΔ160 in NIH3T3 cells lead to tumor formation *in vivo* [97, 106-108], and expression of full length Ron in colon epithelial cells results in an increase in cell motility and invasiveness, while protecting the cells from apoptosis [108]. Silencing Ron expression by RNAi in colon cancer cell lines led to decreased cell proliferation and motility, with an increase in apoptosis [109]. Silencing of Ron also reduced tumorigenesis *in vivo*, suggesting that Ron expression is required to maintain the tumorigenic phenotypes of colon cancer cells [109].

Table 1. Expression of Ron in Primary Human Cancer Tissues

Cancer Type	% of Tumors Expressing Ron	Reference(s)
Breast	50	[87]
	32	[32]
	100	[18]
	8-20*	[38]
Lung	93	[18]
	50 (NSCLC# only)	[103]
Colon	60	[106, 107]
	65	[18]
Pancreatic	93	[114]
	79-93	[115]
	69	[18]
Bladder	33	[116]
Ovarian	56-60	[118]
Prostate	92	[18]
Liver	29 (HCC† only)	[123]
Gastric	73	[18]
Glioblastoma	82	[121]

* MSP/matriptase/Ron co-overexpression
# Non small cell lung carcinoma
† Hepatocellular carcinoma

While mutations in Ron have not been identified in cancers other than in a single lung tumor [110], two alterations have been identified which may have a role in Crohn's disease [111]. A genome wide linkage study performed with a cohort of Crohn's disease patients identified strong linkage disequilibrium with two non-synonymous single nucleotide polymorphisms (SNPs) within the *RON* gene [111]. The first SNP, rs2230590, results in an Arg523Gln substitution while the second, rs1062633, results in a Gly1335Arg substitution. Further evidence for a role for the MSP/Ron pathway in inflammatory bowel diseases (IBD) comes from another genome wide linkage study performed on a cohort of IBD patients. This study identified significant linkage disequilibrium with a SNP located within the *MSP* gene [112]. This nonsynonomous SNP, rs3197999, results in an Arg698Cys coding variant, which is predicted to interfere with the ability of MSP to bind to Ron [112]. Importantly, this coding variant showed association with both Crohn's disease and ulcerative colitis, suggesting that the MSP/RON pathway

may have an important role in multiple forms of inflammatory bowel disease - though the mechanisms for this role have yet to be elucidated [112]. Associations between *RON* and *MSP* SNPs in inflammatory bowel diseases, which predispose patients to colon cancer (for a recent review, see [113]), provide further support for a connection between MSP/Ron function in inflammation and tumor progression (Fig. **1**).

Ron is overexpressed in 79-93% of human pancreatic tissue samples, and in 83% of metastatic lesions [114, 115]. Activation of Ron by MSP in pancreatic cell lines leads to activation of Erk and Akt pathways, as well as induction of EMT characteristics such as increased cell migration, invasion, and loss of E-cadherin [114, 115]. Inhibition of Ron by a neutralizing antibody resulted in inhibition of the cell migratory and invasive phenotypes [115].

Ron is overexpressed in 33% of primary bladder tumors, where Ron levels correlated with poor grade as well as tumor size and stage [116]. Overexpression of Ron in a uroepithelial cell line led to proliferation, motility, and increased survival [116]. Ron also cooperated with Met and EGFR in these cells; co-expression of Ron and Met was significantly associated with decreased survival and metastasis-free survival in 19% of patients [116]. Co-expression of Ron and EGFR was found in 33% of patients and significantly associated with invasion, risk of recurrence, and decreased patient survival [117].

Ron expression was detected in 56% of ovarian cancers and 60% of borderline ovarian tumors [118]. The level of Ron expression also significantly correlated with decreased survival in ovarian cancer patients [119]. A correlation between overexpression of Ron and concomitant expression of Met was demonstrated, and stimulation of ovarian cancer cell lines *in vitro* by MSP and/or HGF lead to increased motility and invasion [118, 120].

Full length Ron, and several splice variants, were expressed in primary human glioblastomas and glioblastoma cell lines. Of the glioblastoma patient samples analyzed, 82% expressed some form of Ron, while 100% of the glioblastoma cell lines analyzed demonstrated Ron expression [121]. MSP was also expressed in glioblastoma cell lines, where it functions to increase cell migration [121]. A novel splice variant, RONΔ90 was also identified, which inhibited MSP-induced phosphorylation of Ron as well as cell migration.

Ron was expressed in 92% of prostate tumor tissues and is overexpressed in prostate cancer cell lines [18]. Ron expression correlates with the stage of disease in the primary tumor and is expressed in prostate metastases. Levels of angiogenic chemokines correlate with Ron expression, and knockdown of Ron resulted in a decrease in angiogenic factors, NF-kB, and endothelial cell migration *in vivo*. Knockdown of Ron also resulted in decreased tumor growth and microvessel density, indicating that Ron may play an important role in the angiogenic process in prostate cancer [122].

Ron has been shown to be overexpressed in two out of seven hepatocellular carcinoma (HCC) tissue samples. The cytokines IL-1α, IL-6, and TNFα, as well as the growth factor HGF were shown to increase Ron expression in a HCC cell line. These factors are commonly upregulated in liver disease and may therefore play a role in liver carcinogenesis through the upregulation of Ron [123]. Notably, the liver is the primary site of MSP production and therefore may contribute to increased Ron activity in the liver.

CONCLUSIONS

In summary, the MSP/Ron pathway appears to be active in a large number of solid tumors from various organs, and MSP/Ron activity correlates with aggressive disease and poor outcome. The known roles for this pathway strongly suggest that MSP/Ron could play a significant, dual role in tumor progression by acting directly on tumor cells and indirectly through inflammatory cells. Thus, inhibition of Ron may provide a promising new avenue for cancer treatment by simultaneously affecting at least two critical aspects of tumor progression. In breast cancer, blockade of Ron function may succeed in decreasing tumor growth, metastasis, and destruction of bones through MSP-driven osteolysis.

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CHAPTER 4

MSP/RON IS A NOVEL MEDIATOR OF OSTEOCLAST ACTIVATION THAT PROMOTES BREAST CANCER-INDUCED BONE DESTRUCTION AND OSTEOPOROSIS

This chapter comprises a manuscript that is currently in revision for publication in Cancer Cell

4.1 Summary

Despite evidence supporting an oncogenic and bone metastatic role in breast cancer, the role of the Ron receptor tyrosine kinase pathway in the bone microenvironment during metastasis remains unknown. Here, I report that the Ron ligand, macrophage-stimulating protein (MSP), is an important contributor to cancer-meditated osteolysis. MSP overexpression leads to a significant increase in the ability of tumor cells to destroy bone and this ability requires Ron activity in host osteoclasts. MSP-driven osteoclast activation does not require RANKL or TGFβ signaling and, importantly, pharmacological inhibition of Ron can prevent both the development of osteolysis and the progression of existing osteolysis. In addition, I demonstrate that the MSP/Ron pathway is pertinent to other cases of pathogenic osteoclast activation; this pathway also functions in bone loss due to osteoporosis. These findings elucidate an important role for the MSP/Ron pathway in osteoclast activation, providing rationale for the use of Ron inhibitors for treatment of bone destruction in the setting of metastatic cancer or osteoporosis.

4.2 Highlights

- MSP promotes osteolytic bone destruction
- Activation of osteoclasts by MSP/Ron does not depend on RANKL or TGFβ signaling
- Lack of Ron tyrosine kinase prevents osteoporosis-mediated bone loss
- Ron inhibitors can prevent cancer-induced osteolysis and osteoporosis

4.3 Significance

My study reveals a new mechanism by which osteoclasts become activated *in vivo*. Activation of Ron tyrosine kinase leads to osteoclast activity and bone resorption through a mechanism that is not dependent on RANKL, which is widely recognized as a key regulator of pathogenic osteoclast activity. I have also discovered a role for the MSP/Ron pathway in osteoporosis-related bone loss, suggesting that the pathogenic effects of MSP/Ron signaling in bone are not limited to cancer. Importantly, pharmacological inhibition of Ron reduced both MSP-mediated tumor osteolysis and osteoporotic bone loss, providing preclinical support for the use of Ron inhibitors as therapeutic agents against pathological bone loss.

4.4 Introduction

Macrophage-stimulating protein (MSP, also known as human MST1 or mouse HGFL; hepatocyte growth factor like) is a plasminogen-related growth factor that was originally identified as a serum protein that caused macrophage chemotaxis and activation. MSP is secreted as an inactive single-chain precursor (pro-MSP), which becomes active after proteolytic cleavage, forming a disulfide-linked heterodimer (Danilkovitch and Leonard 1999). The first protease identified to activate pro-MSP under biological conditions was membrane-type serine protease 1 (MT-SP1, also known as ST14 or matriptase) (Bhatt 2007). However, it is now apparent that there are other proteases capable of pro-MSP maturation such as hepsin, human airway trypsin-like protease (HAT), and hepatocyte

growth factor activator (HGFA) (Ganesan 2011; Kawaguchi 2009; Orikawa 2012). MSP exerts several important biological effects depending on the cell type in which its receptor, macrophage-stimulating-1 receptor (MST1R, or Ron), becomes activated. MSP is the only known ligand for Ron, and Ron is one of only two members of a distinct receptor tyrosine kinase family that also includes Met (Wang 1995). Ron is expressed in many tissues during development; however, in adults its expression is restricted to certain epithelial-derived cells (such as keratinocytes), nociceptive neurons, resident tissue macrophages, and osteoclasts. MSP can promote migration and survival of epithelial cell lines and can promote an epithelial-to-mesenchymal transition in immortalized canine kidney cells *in vitro* (Cote 2007; Pagan 1999; Wang 1996).

In addition to the effects the MSP/Ron pathway has in a normal cellular context, evidence now suggests an important role in the cancer setting. Ron is expressed at high levels in many different epithelial cancers as well as malignancies of brain and bone (Kretschmann 2010). MSP and Ron are coordinately overexpressed in over 20% of human breast cancers, and their overexpression along with matriptase has been shown to be an independent prognostic indicator for metastasis and poor survival (Welm 2007). Overexpression of Ron in breast epithelial cells leads to the transformation of cell lines and metastatic tumor development in mice (Feres 2009; Zinser; 2006). Overexpression of MSP in tumor cells driven by the polyomavirus middle T antigen under the control of the mouse mammary tumor virus promoter (MMTV-PyMT) led to spontaneous metastasis to bone and a significant increase in

metastasis to other organs (Welm 2007). The fact that MSP was sufficient to promote spontaneous metastasis to bone is noteworthy, as spontaneous bone metastasis has proven very difficult to achieve in genetically engineered mouse models. The bone metastases that developed in this model were osteolytic, replicating what is most commonly seen in human breast cancer patients. Interestingly, the overexpression of Ron in mammary tumors did not lead to an increase in bone metastasis (Zinser 2006; A.L.W. unpublished data), suggesting a non-cell autonomous mechanism where tumor-host interactions may play a key role in MSP-driven bone metastasis.

Over 70% of patients with metastatic breast cancer have bone metastases. These metastases cause a number of complications, including severe pain, nerve compression, hypercalcemia, and debilitating bone fractures (Colman 2002; Deil 2000). The development and growth of bone metastases depend on the interactions between tumor cells and cells within the bone microenvironment. Bone homeostasis is normally maintained by balanced osteoclast and osteoblast activities, and it is the ability of tumor cells to disrupt this delicate balance that results in bone destruction and metastatic tumor growth. The “vicious cycle” hypothesis describes the complex interactions that result in bone destruction and subsequent metastatic tumor growth: As active osteoclasts resorb bone, growth factors such as TGFβ are released from the bone matrix into the bone-tumor microenvironment. Such growth factors not only increase survival and proliferation of the metastatic tumor cells, but also lead to production of factors such as parathyroid hormone-related peptide and

interleukin 11. These proteins indirectly lead to further osteoclast differentiation and activation by stimulating secretion of receptor activator of nuclear factor-kB ligand (RANKL) from osteoblasts. RANKL is a key mediator of osteoclastogenesis and osteoclast activity, thus completing the cycle of bone resorption and metastatic tumor growth (Guise and Mundy 1998; Weilbaecher 2011).

Ron is expressed in osteoclasts and expression of Ron increases dramatically during osteoclast differentiation (Yang 2008). In addition, MSP has been shown to cause activation, but not differentiation, of osteoclasts *in vitro* (Kurihara 1996; Kurihara 1998). This suggests that the ability of MSP to drive osteolytic bone metastasis (Welm 2007) may be due to a favorable interaction of MSP with differentiated, mature osteoclasts, but such a role has never been elucidated *in vivo*. Although several molecular contributors of bone metastasis have been identified, of which RANKL is key, identification of other targetable pathways may lead to additional therapies and/or more effective therapeutic combinations.

4.5 Results

4.5.1 Ron expression in the host is required for MSP-driven breast cancer osteolysis

We have previously shown that MSP drives spontaneous osteolytic bone metastasis (Welm 2007). To investigate the hypothesis that MSP-mediated bone metastasis depends on non-cell-autonomous activation of Ron, we injected

PyMT or PyMT-MSP tumor cells (Welm 2007) into the tibias of wild-type (WT) mice or mice in which the tyrosine kinase domain of Ron has been deleted from the genome (RonTK -/-) (Waltz 2001). Expression of MSP in the tumor cells significantly increased the ability of these tumors to induce osteolysis in WT bones (Figure 4.1A). In contrast, when tumors overexpressing MSP were injected into RonTK-/- bones, they induced very little osteolysis. This was comparable to the basal levels of osteolysis induced in control tumor cells (Figures 4.1A and 4.1B). MSP-driven osteolysis could not be explained by altered tumor growth in the bone, as no statistically significant differences in tumor size or proliferation were observed between cohorts (Figures 4.1C and 4.1D). Thus, Ron activity in the host was required for the ability of MSP-expressing tumor cells to induce osteolysis in the bone microenvironment. The presence of residual tumor-induced osteolysis caused by control tumors both in WT and RonTK -/- mice demonstrates some degree of osteolysis that is independent of the MSP/Ron pathway, likely RANKL-mediated (addressed below).

To determine if MSP-driven osteolysis was due to an increase in the number of osteoclasts present within the microenvironment, bone sections were stained for the osteoclast-specific protein tartrate resistant acid phosphatase (TRAP) and the number of TRAP-positive (TRAP+) multinuclear cells were quantified. We detected no significant difference in the number of TRAP+ osteoclasts when comparing bones harboring control tumors versus MSP expressing tumors (Figures 4.1E and 4.1F). We also examined normal, non-

tumor-bearing bones to determine whether the RonTK-/- mice displayed a defect in osteoclastogenesis. Again, there was no difference in the number of osteoclasts present in the bones of RonTK -/- mice when compared to WT mice (Figures 4.1E and 4.S1A).

To determine if protection from osteolysis in the Ron TK-/- mice was due to an increase in bone formation due to elevated osteoblast activity, WT and Ron TK-/- bones were labeled *in vivo* and histomorphometric bone parameters were analyzed. There were no significant differences in the mineral apposition rate (MAR) or the bone formation rate (BFR), indicating that there is no inherent difference in osteoblast activity between WT and Ron TK-/- mice (Figures 4.S1B-D). To ensure that a tyrosine kinase-independent function of Ron was not contributing to osteoblast activity, we also examined bones of mice completely lacking murine Ron (STK) protein (Correll 1997). Again, no differences in osteoblast activity were seen between WT and total Ron knockout mice (STK-/-) (Figures 4.S1B, 4.S1E-F). Consistent with this finding, staining bone sections with antibodies specific for Ron showed expression in osteoclasts and chondrocytes, but not in osteoblasts (Figure 4.S1G), and no Ron or phosphorylated Ron expression was detected in the MC3T3-E1 osteoblast cell line (Figure 4.S1H). Together, these data strongly suggest that the ability of MSP-expressing tumor cells to induce osteolysis was not due to changes in osteoclast numbers or osteoblast activity. We therefore pursued the hypothesis that MSP-mediated osteolysis was due to Ron-dependent effects on osteoclast activity.

4.5.2 Host Ron activity drives osteolysis from metastatic human breast cancer

To ensure that our findings were not restricted to the effects of mouse tumors or tumors engineered to overexpress MSP, we examined the expression levels of MSP, Ron, and matriptase in a panel of human breast cancer cell lines and chose a cell line (DU4475), which expressed each of these genes above an average expression level, similar to our previous report on patient tumor samples (Figure 4.S2A; Welm 2007). The metastatic breast cancer cell line DU4475 was selected and injected into the tibias of WT or RonTK-/- mice (both crossed with NOD.SCID and then backcrossed into the FVB genetic background). While the DU4475 cell line was osteolytic in NOD.SCID mice carrying wild-type alleles of Ron (NOD.SCID/RonTK+/+), osteolysis was significantly reduced in the NOD.SCID/RonTK-/- mice (Figures 4.2A and 4.2B). These results show that MSP/Ron-dependent osteolysis is not restricted to the mouse overexpression model, but can be expanded to include human cell lines that naturally overexpress endogenous MSP. Again, there was no significant difference in the number of TRAP+ osteoclasts in NOD.SCID/RonTK+/+ versus NOD.SCID/RonTK-/- mice harboring DU4475 tumors (Figures 4.2C and 4.2D) or in normal, non-tumor-bearing NOD.SCID/RonTK+/+ and NOD.SCID/RonTK-/- mice (Figures 4.S2B and 4.S2C). Again, the lack of osteolysis could not be explained by a decrease in tumor proliferation in the NOD.SCID/Ron TK-/- mice (Figure 4.2E).

4.5.3 T cells are dispensable for MSP-induced osteolysis

Because the above xenograft experiments had to be performed in immune-compromised mice, and because T cells have previously been shown to influence osteoclast activity (Takayanagi 2012), we wanted to ensure that the lack of tumor-induced osteolysis from human DU4475 cells in NOD.SCID/RonTK-/- mice was not due to a combination effect of Ron TK deletion and the compromised immune system. To test whether T cells were required for MSP/Ron-dependent osteolysis, we compared the effects of injecting PyMT-MSP tumor cells into the tibias of syngenic immune-competent (FVB/RonTK+/+ or FVB/RonTK-/-), or immune-compromised (NOD.SCID/RonTK+/+ or NOD.SCID/RonTK-/-) mice. MSP-mediated osteolysis was not significantly altered in the immune-compromised mice and still demonstrated the necessity for Ron activity in the host (Figures 4.S2D and 4.S2E). There was also no significant difference in tumor growth or in the number of TRAP+ osteoclasts in these experiments (Figures 4.S2F-H). While not statistically significant, there was a trend toward increased growth in the immune-deficient background, but no difference in osteolysis. These data show that the mechanism for MSP-driven bone destruction does not require T-cell activity, or any other component of the adaptive immune system.

4.5.4 Treatment with Ron inhibitors blocks osteolysis

Based on the data obtained using RonTK-/- mice, we tested whether pharmacological inhibition of Ron could provide protection from osteolysis. We

injected tibias with MSP-expressing PyMT tumor cells or DU4475 breast cancer cells and treated them with two different small molecule tyrosine kinase inhibitors that are selective for Ron and Met (OSI-296 and ASLAN002). OSI-296 is more selective for Met (IC_{50} of 42 nM for Met and 200 nM for Ron; Steinig 2013), while ASLAN002 (also known as BMS-777607) is more selective for Ron (IC_{50} of 3.9 nM for Met and 1.8 nM for Ron; Schroeder 2009). When animals were treated with either drug, beginning 3 days after tumor injection, osteolysis from PyMT-MSP tumors was significantly inhibited, phenocopying the results obtained with RonTK-/- mice. OSI-296 caused an average 4-6 fold inhibition of osteolysis and ASLAN002 caused 6-10 fold inhibition of osteolysis (Figures 4.3A-D). ASLAN002, but not OSI-296, also prevented osteolysis from DU4475 cells (Figures 4.3E and 4.3F). Ron inhibition did not have an effect on the ability of tumors that do not express MSP to cause osteolysis, again implicating an osteolytic pathway that is independent of MSP/Ron signaling (Figures 4.S3A and 4.S3B). To determine the potential of Ron inhibitors in a more clinically relevant setting, we allowed the animals to develop osteolysis that was visible by X-ray in live animals, and then began treatment (this was, on average, 3 weeks after tumor injection). While the osteolysis in vehicle treated mice continued to progress, Ron inhibition was able to prevent further tumor-induced bone loss. These results carry clinical relevance, as they suggest that Ron inhibitors could be useful in the postmetastatic, post-osteolytic setting (Figures 4.3A and 4.3C). Treatment with OSI-296 also reduced PyMT-MSP tumor growth and proliferation rate in the bone, while treatment with ASLAN002 showed a trend in decreased

growth, but did not reach statistical significance (Figures 4.S3D-F). Neither drug significantly inhibited growth of DU4475 tumors (Figure 4.S3G). Again, Ron inhibition had no significant effect on the number of TRAP+ osteoclasts present (Figures 4.S4A and 4.S4B). Ron inhibitors also did not affect RANKL production as measured by serum ELISA, suggesting that the lack of bone destruction was not due to indirect inhibition of RANKL signaling (Figure 4.3G). Ron inhibitors also did not affect bone formation *in vivo,* supporting a model in which blockade of Ron signaling leads to a specific reduction in osteoclast activity, not an increase in osteoblast activity (Figures 4.S4C-S4E).

4.5.5 MSP/Ron signaling is a novel mechanism of osteolysis that is not dependent on RANKL or TGFβ signaling

Both RANKL and TGFβ have been shown to play major roles in osteoclast activation and the "vicious cycle." Denosumab, a RANKL antagonist, was recently FDA-approved to treat bone metastases and osteoporosis based on demonstration of increased time to development of skeletal-related events, such as fracture, in patients (Cummings 2009; Papapoulos 2012; Stopeck 2010). Mechanistically, we wanted to determine if MSP/Ron functionally interacts with RANKL and/or TGFβ pathways. To test whether MSP-driven osteolysis is dependent on activation of RANK by RANKL, we treated mice with the murine RANKL antagonist, muRANK-Fc, 3 days after intratibial injection of tumor cells. As expected based on existing literature (Canon 2008; Holland 2010), destruction of bone in PyMT control tumors was significantly impaired upon

treatment with the RANKL antagonist (Figures 4.4A and 4.4B), despite no significant effect on tumor growth (Figure 4.S5A). These data support the observation that the low level of Ron-independent osteolysis present in control tumors is dependent upon the RANKL pathway and, importantly, that the antagonist is functional in our assays. However, PyMT tumor cells overexpressing MSP were able to cause significant osteolysis even in the presence of the RANKL antagonist. In addition, although the levels of MSP-driven osteolysis in the Ron TK-/- mice were already very low, inhibition of RANKL did not reduce this further (Figures 4.4A and 4.4B). Staining of sections from these samples demonstrated successful RANKL inhibition by the lack of TRAP+ cells in these groups, as expected from published literature demonstrating that TRAP expression is regulated downstream of RANK (Canon et al., 2008) (Figure 4.4C). However, osteoclasts were still present following RANKL inhibition, as demonstrated by NFATc1 and Ron immuno-staining (Figure 4.S5C). These data suggest that although the RANKL antagonist is effective in our assays, the ability of MSP to induce osteolysis does not require active RANKL signaling.

The TGFβ pathway has also been shown to be an integral part of the “vicious cycle.” TGFβ is released from the bone matrix by osteoclasts and binds to its receptor on tumor cells, stimulating production of pro-osteoclastogenic cytokines. Mechanisms of osteolysis involving TGFβ that are potentially RANKL-independent have been suggested (Itonaga 2004; Sethi 2011); therefore, it is possible that MSP/Ron functionally interacts with the TGFβ pathway to induce

osteoclast activation. To test this possibility, we utilized PyMT tumor cells in which the TGFβ type-II receptor (TGFβRII) had been deleted using a Cre-lox system (Forrester 2005). We then overexpressed MSP and injected these cells into WT mice. While the absence of TGFβ signaling did not affect tumor growth (Figure 4.S5D), it did affect the ability of control tumors to induce basal levels of osteolysis (Figures 4.4D and 4.4E). Similar to RANKL inhibition, TGFβRII loss in the tumor cells impaired the ability of control tumors to induce osteolysis. This again supports the notion that the basal level of osteolysis induced by PyMT control tumor cells is dependent on RANKL and TGFβ signaling. Tumors overexpressing MSP, in contrast, caused robust osteolysis regardless of whether they expressed TGFβRII. Taken together, this evidence suggests that, while the RANKL-TGFβ portion of the vicious cycle is active in the PyMT model of bone metastasis, MSP/Ron activates osteoclasts through a mechanism that does not depend on RANKL or TGFβ.

To further test the hypothesis that MSP/Ron is sufficient to stimulate osteolysis in a manner that does not rely on the RANKL pathway, we utilized another human breast cancer model (MDA-MB-231), which has been previously shown to be highly dependent on RANKL for osteolysis (Canon 2008). Concordant with this, MDA-MB-231 cells do not express MSP at detectable levels (Figure 4.5A), allowing us to assess the consequences of MSP overexpression, which occurs in approximately 40% of human breast cancers (Welm et al., 2007). We overexpressed MSP in this cell line and injected these cells into the tibias of NOD.SCID mice. The expression of MSP led to a

significant increase in the ability of these cells to induce osteolysis (Figures 4.5B and 4.5C). While treatment with the RANKL antagonist again reduced osteolysis from control tumor cells, it did not significantly reduce the osteolysis from MSP-expressing tumor cells. Treatment with OSI-296, in contrast, was able to reduce MSP-mediated osteolysis to levels seen in control tumor cells. These data demonstrate that MSP gain-of-function can significantly increase osteolysis through a mechanism that can override RANKL dependency. Taken together, our data strongly suggest that the MSP/Ron pathway is a novel mediator of osteoclast activity in bone-metastatic breast cancer.

4.5.6 MSP/Ron signaling promotes bone degradation by stimulating RANK-independent osteoclast survival, c-Src phosphorylation, and osteolytic activity

To determine the cellular mechanism by which Ron signaling functions in specific steps during osteoclastogenesis and osteoclast activation, we analyzed the effect of MSP on osteoclasts *in vitro*. Differentiated osteoclasts were stimulated with recombinant MSP, and their ability to resorb an artificial bone matrix was analyzed. Addition of MSP to WT osteoclasts led to a significant increase in the resorptive capacity of these cells, while having no effect on RonTK-/- osteoclasts (Figures 4.6A and 4.S6A). This increase in osteoclast resorption was only slightly reduced upon addition of the RANKL antagonist (Figure 4.6B), but was dramatically reduced upon addition of Ron inhibitors ASLAN002 or OSI-296 (Figures 4.6C and 4.S6B). Simultaneous treatment with

the RANKL antagonist and either of the Ron inhibitors reduced osteoclast activity to a greater extent than either treatment alone (Figures 4.6C and 4.S6B), again suggesting that both Ron and RANKL contribute to bone destruction, but do so through separate pathways in osteoclast activation.

As expected from our observations *in vivo*, MSP stimulation did not increase osteoclast differentiation, and even demonstrated an inhibitory effect when added at early time points relative to the onset of differentiation (Figures 4.S6C and 4.S6D). MSP was not sufficient for differentiation when added at any time during the differentiation process (Figure 4.S6E). This suggests that RANKL is required to prime these cells for cell fate determination and differentiation, similar to that of other factors which have been shown to activate osteoclasts (Itoh 2001; Ha 2011). The ability of these cells to resorb matrix as they differentiated was also tested. Interestingly, MSP was able to stimulate osteoclast resorption of RANKL-differentiated osteoclast precursor cells, even when added at very early time points (Figure 4.S6F). Together, these data suggest that RANKL is required for osteoclast differentiation, but can be dispensable for the resorptive activity of fully-differentiated osteoclasts when MSP is present.

Because RANK signaling is thought to be required for osteoclast survival, we also questioned whether MSP could affect the survival of existing osteoclasts in the absence of RANKL. To determine this, osteoclasts were fully differentiated in the presence of M-CSF and RANKL. These factors were then removed from the culture and MSP was added (see schematic in Figure 4.S6G). The cells were

cultured for an additional 48 hours and osteoclast survival was determined by counting the number of TRAP+ cells present. While the number of surviving osteoclasts was reduced in all conditions tested without RANKL and M-CSF, the number of surviving osteoclasts was significantly augmented in the presence of MSP (Figure 4.6D). These experiments demonstrate that the MSP/Ron pathway does not promote osteoclast differentiation, but instead functions to stimulate osteoclast activity and survival.

c-Src has been shown to be critical in the regulation of osteoclast survival, motility, cytoskeletal reorganization, and resorption ability (Boyce 1992; Izawa 2012; Soriano 1991). To determine whether MSP/Ron signaling stimulates c-Src activity independently of RANK, we tested c-Src phosphorylation in the presence or absence of RANKL signaling. Addition of the RANKL antagonist to WT osteoclasts resulted in a decrease in phosphorylated c-Src. However, MSP was able to rescue the loss of phosphorylated c-Src, indicating that the MSP/Ron pathway functions as an alternative pathway of c-Src activation in the absence of RANKL signaling (Figures 4.6E and 4.6F). Taken together, these results suggest that Ron plays a critical role in a signaling pathway that is known to be essential for osteoclast activity (Figure 4.S6H).

4.5.7 Ron expression in the host is required for ovariectomy-induced bone loss

Pathways involved in tumor-driven osteolysis are also often implicated in other pathologies leading to bone destruction. To investigate the role of the

MSP/Ron pathway in bone loss due to postmenopausal osteoporosis, we performed ovariectomies in WT and RonTK-/- mice and analyzed bone loss by dual-energy X-ray absorptiometry (DXA) and bone histomorphometry 28 days later. The ovarectomized RonTK-/- mice were more resistant to loss of bone mineral density (BMD), bone volume, and trabecular thickness, with a concomitant decrease in trabecular spacing as compared to WT mice or sham-operated controls (Figures 4.7A-E). In fact, loss of Ron resulted in complete protection from osteoporosis, as RonTK-/- bones were not statistically different than sham-operated controls. There also was no difference in the bone histomorphometric parameters between sham-operated mice of either genotype, demonstrating that there are no basal differences in bone density in the Ron TK-/- mice under normal physiologic conditions. Importantly, treatment with ASLAN002 after ovary removal also completely prevented bone loss (Figures 4.7A-E). These data further show that MSP/Ron activity is a critical pathway for osteoclast activation both in physiological and pathological states.

4.6 Discussion

4.6.1 Importance of MSP/Ron in human breast cancer

Previous studies have indicated an important role for the MSP/Ron pathway in human breast cancer, as well as in many other cancers (Kretschmann 2010). Expression of components of the MSP/Ron pathway are highly significant, independent prognostic factors for metastasis and death in breast cancer patients. A retrospective study on sites of metastasis in women

whose tumors overexpress the MSP/Ron pathway indicated that they have a significant increase in metastasis overall, as well as increased metastasis to the bone (Welm 2007). In fact, MSP is overexpressed in approximately 40% of breast cancers, indicating a substantial number of patients whose tumors have the potential for an interaction with Ron in the microenvironment leading to metastasis. Despite the importance of this pathway in breast cancer metastasis, little was understood regarding the ability of this pathway to promote bone metastasis - the most common site of metastasis in patients.

In this study, we show that expression of MSP in breast cancer cells promotes tumor-induced osteolysis through activation of Ron in the host osteoclasts. The bone destruction caused by MSP-expressing tumors was not affected by inhibition of RANKL or TGFβ signaling, indicating a novel pathway of osteoclast activation. Additionally, we have demonstrated the potential for therapeutically targeting the MSP/Ron pathway; inhibition of Ron with two inhibitors and in several preclinical mouse models blocked tumor-induced bone destruction, even after osteolytic disease had been well established. Genetic deletion or pharmacological inhibition of Ron activity was also able to reduce the development of osteoporosis-associated bone loss, suggesting that therapeutically targeting this pathway could be valuable in multiple diseases involving bone loss.

4.6.2 Role of Ron in the osteoclast

Ron expression in differentiated osteoclasts, and the dramatic increase in Ron mRNA expression as pre-osteoclasts differentiate into osteoclasts, was demonstrated several years ago (Yang 2008). However, the function of Ron in these cells was unknown. We have shown that Ron activation by MSP stimulates osteoclast survival and bone resorption. In addition, we have shown that Ron activity in osteoclasts leads to activation of c-Src and can function as an alternative pathway to c-Src activation in the absence of functional RANKL signaling. c-Src is responsible for several critical functions in the osteoclast, including cytoskeletal reorganization and the formation of the ruffled border, thereby fundamentally regulating the ability of an osteoclast to resorb bone (Boyce 1992). Deletion of c-Src in mice leads to severe osteopetrosis, despite the presence of mature osteoclasts, indicating that the disease developed due to a defect in osteoclast function and not a lack of differentiation (Soriano 1991). Similarly, the Ron TK-/- mice do not demonstrate a reduction in the number of osteoclasts but, under diseased conditions, demonstrate a defect in osteoclast function. This distinguishes the MSP/Ron pathway from others, such as RANKL, which demonstrate a combined effect on both differentiation and resorptive activity.

In addition to promoting survival, RANK drives two key pathways in osteoclasts: differentiation and activation. RANK associates with c-Src and, together with αvβ3, leads to cytoskeletal reorganization and resorption. This function of RANK is independent of its ability to regulate osteoclast differentiation

(Izawa 2012). Based on our data, it is likely that Ron functions to activate c-Src in the absence of functional RANK signaling, while having no effect on RANK-regulated pathways of differentiation. Although not critical during bone development (Ron TK-/- mice have normal bone histomorphometric parameters), the effects of aberrant Ron activation in osteoclasts become readily apparent in disease settings. This suggests that, while the MSP/Ron pathway is not absolutely necessary for osteoclast function, this pathway plays an important role in the modulation of osteoclast activity in the cancer setting and in the development of osteoporosis.

4.6.3 Association of the MSP/Ron pathway with the vicious cycle

The role of both RANKL and TGFβ in the “vicious cycle” is a well-established mechanism for osteoclast activation and has been shown to be crucial for the ability of many breast cancer cells to induce bone loss. Our data indicate that the MSP/Ron pathway functions in parallel to the RANKL and TGFβ pathways. While it is still a possibility that these pathways interact in some fashion, the evidence suggests that, in terms of their ability to activate osteoclasts, they function as separate, additive pathways in osteoclast activation. For example, control PyMT tumors and MDA-MB-231 cells demonstrated dependence on RANKL and TGFβ for their ability to induce osteolysis. However, when MSP is expressed (exogenously in PyMT tumors or MDA-MB-231 cells, or endogenously in DU4455 cells), the MSP/Ron pathway can bypass the previously described pathways and become the dominant force driving bone

destruction (Figure 4.6H). It will be interesting to further understand the interplay of these pathways in breast cancer-induced bone loss as well as in other bone diseases.

4.6.4 Potential for Ron inhibitors as therapy against osteolysis

Inhibition of the Ron pathway can be achieved through pharmacological inhibition of its tyrosine kinase activity, either directly with small molecule inhibitors or indirectly through ligand-blocking antibody approaches (Kretschmann 2010). It is not yet clear if both strategies of inhibition will prove beneficial, as Ron is known to have multiple isoforms that function in a ligand-independent manner and may not be inhibited by ligand-blocking antibodies. Although several small molecule Ron kinase inhibitors are selective for Ron, most also inhibit the related Met kinase. Several of these Ron/Met inhibitors are being developed as potential anticancer agents and the Ron-selective ASLAN002 compound is currently in a Phase I clinical trial (NCT01721148) for metastatic cancer. Here we have shown that two small molecule inhibitors for Ron/Met, including ASLAN002, prevent osteolysis in several metastatic breast cancer models, even after osteolysis had initiated. The blockade in osteolysis phenocopied that seen in the RonTK-/- mice, strongly suggesting that the effect is through Ron inhibition. It will be interesting to determine whether combinatorial inhibition of Ron and RANKL would lead to an additive or synergistic effect in inhibiting tumor-induced osteolysis in patients, as we have demonstrated *in vitro*.

We focused on breast cancer because it is the most common cause of osteolytic bone metastasis. However, we also note that MSP is specifically overexpressed in others cancers that are often metastatic to bone and osteolytic, such as lung cancer and multiple myeloma (Figures 4.S7A and 4.S7B). Therefore, Ron inhibitors may be valuable as therapeutic agents in cancers other than those arising in the breast – perhaps in combination with other modulators of osteoclast activity such as denosumab or bisphosphonates (Lipton et al., 2012). Moreover, as is the case with other inhibitors of osteoclast activity, Ron inhibitors may be applicable to other diseases that cause bone loss. Indeed, we have demonstrated that Ron inhibition was able to significantly reduce bone loss due to osteoporosis.

4.7 Experimental procedures

4.7.1 Tumor injections and X-ray analysis

All procedures involving mice and experimental protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Utah. The Ron TK-/- mice, which reside on the FVB background, were crossed with NOD.SCID mice for an immune-compromised mouse model. As a control, wild-type FVB mice were also crossed with NOD.SCID mice. The STK-/- mice, which are on a C57BL/6 background, were compared to wild-type C57BL/6 mice as controls. Mice for all experiments were 6 to 8 weeks of age. For intratibial injections, 1 x 10^5 (PyMT or DU4475) or 3 x 10^5 (MDA-MB-231) tumor cells were resuspended in 10 µL matrigel and injected into the right tibia of anesthetized

female FVB (PyMT) or NOD.SCID mice carrying various alleles of Ron. Osteolysis was assessed by X-ray radiography. Dissected hindlimbs from mice sacrificed at the final time point in each experiment were placed in the Kodak In-vivo Multispectral Imaging System FX and exposed to X-ray radiography at 35 kV for 100 sec. Excess tumor tissue was removed from the bones prior to X-ray. Osteolytic lesions were quantified using Image J software (NCBI). All images were compared to the contralateral leg as an internal control.

4.7.2 Microcomputed tomography imaging and bone mineral density measurements

Representative samples were imaged by microcomputed tomography (μCT, Skyscan) with volume rendering snapshots of the tibia. For the osteoporosis study, representative samples were imaged and presented as volume rendering snapshots of the axial view of the tibial metaphysis region. For bone mineral density (BMD) measurements, the right tibia was removed and fixed in 10% NBF overnight. The tibia were then scanned by dual-energy X-ray absorptiometry (DXA) using a Norland pDEXA densitometer (Norland Medical Systems). A region including the primary and secondary spongiosa was used to determine the BMD of the tibia.

4.7.3 Tissue processing and immunohistochemical staining

Hindlimb bones were excised from mice at the time of sacrifice and bones were fixed in 10% neutral-buffered formalin, decalcified in Formical-2000 (Decal

Corp.) for 8 days and embedded in paraffin for hematoxylin and eosin (H & E) or immunohistochemical staining. Osteoclast number was assessed as $TRAP^+$ multinucleated cells containing 4 or more nuclei and reported as $TRAP^+$ cells per field. Three sections per mouse and 5 fields per section were used for quantification with fields representing the entire length of the tibia. Tumor proliferation rate was reported as the number of cells which were positive for phospho-H3 staining compared to the number of unstained cells per field expressed as a percent. Three sections per mouse and 5 fields per section were used for quantification with analysis being restricted to within the bone marrow cavity. Immunohistochemical analysis was performed with heat-induced antigen retrieval in sodium-citrate buffer (Dako). Primary antibodies used were anti-TRAP at 1:50 (Santa Cruz, sc-30833), anti-Ki67 at 1:100 (Santa Cruz, sc-12202S), anti-phosphoH3 at 1:100 (Cell Signaling, #9701), anti-NFATc1 at 1:50 (Santa Cruz, sc-13033), and anti-Ron at 1:250 (Santa Cruz, sc-322). Bones from mice lacking Ron were used as a negative control (Correll, 1997). Biotinylated secondary antibody was used with the EnVision$^+$ system HRP kit (Dako) and nuclei counterstained with hematoxylin.

4.7.4 Osteoclast resorption assay

Bone marrow osteoclasts were generated from 6- to 8-week-old WT and Ron TK-/- mice. Briefly, bone marrow was isolated from hindlimb bones and plated in α-MEM media containing 10% heat-inactivated fetal bovine serum (FBS, Sigma), 100 units/mL penicillin-streptomycin (Hyclone), and 33 ng/mL

human macrophage colony-stimulating factor (hM-CSF) (PeproTech) for 24 hours. Nonadherent cells were collected and frozen for later use. To assess resorption activity, bone marrow precursor cells were thawed and seeded at a density of 1 x 10^5 cells per well in BioCoat Osteologic slides (BD Biosciences) in α-MEM media containing 10% FBS and antibiotics. After adhering overnight, media was removed and media containing 33 ng/mL hM-CSF and 9 ng/mL muRANKL (R & D systems) was added. The medium was changed every 3 days depending on acidification, and the assay was terminated as indicated. Recombinant human MSP was prepared and added daily as indicated. Cells were removed with 6% bleach and slides were viewed microscopically using phase-contrast with 20x magnification. Osteolytic resorption area within 5 representative fields was determined using Image J software. Experiments were performed in triplicate. Recombinant MSP was prepared as follows (to be described in detail elsewhere): Concentrated, serum-free media from an MCF7 cell line engineered to overexpress MSP was cleaved with kallikrein (Invitrogen; 4 ng of kallikrein/1 ug of supernatant protein at 37^{0}C for 2.5 hours). The reaction was diluted to 20 mL with 10 mM sodium phosphate, pH7.4 and loaded onto a 5 mL HiTrap heparin column (Millipore) equilibrated with 20 mL of 10 mM sodium phosphate, pH7.4, at a rate of 1mL/min. The column was washed with 10mL of 10mM sodium phosphate, pH7.4, at a rate of 1mL/min. The bound protein was eluted with 7mL of serial concentrations of NaCl in 10 mM sodium phosphate, pH7.4: 0.1M, 0.15M, 0.2M, 0.25M, 0.3M, 0.35M, 0.4M, 0.45M, and 2M. Each fraction was collected and concentrated/desalted on a spin column. Each fraction

was run for MSP western analysis and ELISA analysis to determine purity and concentration.

4.7.5 Osteoclast survival assay

Bone marrow precursor cells were thawed and seeded at a density of 2 x 10^5 cells per well in a 24-well plate containing glass coverslips in α-MEM media containing 10% FBS and antibiotics. After adhering overnight, media was removed and media containing 33 ng/mL hM-CSF and 9 ng/mL muRANKL was added. Cells were allowed to differentiate for 7 days with media changed every 3 days. Coverslips were then imaged microscopically using phase contrast to determine osteoclast number in each well. To test survival, factor-containing media was removed and α-MEM media containing 10% FBS was added as well as MSP as indicated. Cells were cultured for an additional 48 hours, fixed with 10% NBF for 10 minutes, and washed with 1x PBS. For TRAP fluorescent staining, the leukocyte acid phosphatase kit (Sigma) was used according to the manufacturer's instructions, with the substitution of 200 μM ELF97 (Invitrogen) as a phosphatase substrate. Coverslips were incubated in TRAP solution for 15 minutes at 37 degrees, washed with 1x PBS, and counterstained with DAPI. The number of multinucleated TRAP+ cells (containing 4 or more nuclei) within 5 representative fields were included for quantification. Experiments were performed in triplicate.

4.7.6 Murine RANKL ELISA

Quantitative levels of murine RANKL in serum isolated from mice at the end-point of experiments was determined in triplicate by ELISA according to the manufacturer's protocol (Quantikine immunoassay kit, R & D systems).

4.7.7 Pharmacological inhibition *in vitro* and *in vivo*

For *in vitro* experiments, OSI-296 (OSI pharmaceuticals) and ASLAN002 (BMS-777607; Selleck) were dissolved in DMSO. Inhibitors were added to *in vitro* osteoclast assays at a final concentration of 10 µM daily for 3 days beginning on day 9 of the experiment. muRANK-Fc (Amgen) was provided as a stock of 4.1 mg/mL in PBS and was added to *in vitro* assays at a final concentration of 10 µg/mL daily as indicated.

For *in vivo* experiments, the OSI-296 inhibitor was dissolved in 40% Trappsol with 0.01M HCl and mice were treated at a concentration of 200 mg/kg daily by oral gavage. Treatment began either 3 days after tumor cell injection for prophylactic experiments, or 3 weeks after tumor cell injection for therapeutic experiments. ASLAN002 was dissolved in 70% PEG400 with 1x PBS and mice were treated at a concentration of 50 mg/kg daily by oral gavage. muRANK-Fc was given at a concentration of 10 mg/kg every 3 days by intraperitoneal injection. For the osteoporosis study, mice were treated beginning on the day after ovary removal. The treatment regimen for OSI-296 was 200 mg/kg every other day by oral gavage. ASLAN002 was administered at 50 mg/kg every other day by oral gavage. Treatment continued until the time of sacrifice at 28 days.

4.7.8 Cell culture

The DU4475 cell line (ATCC) was maintained in RPMI (Hyclone) with 10% FBS and antibiotics. The MDA-MB-231 cell line (ATCC) was maintained in RPMI with 10% FBS, antibiotics, and HEPES (Sigma). MC3T3-E1 subclone 4 cells (ATCC) were maintained in α-MEM with 10% FBS and antibiotics. PyMT tumors were isolated and transduced as previously described and frozen for later use (Welm et al., 2005; Welm et al., 2007). Cells were thawed and grown for 2 to 3 days in DMEM/F12 with 10% FBS, antibiotics, insulin (Gibco), hydrocortisone, and epidermal growth factor prior to injections. MDA-MB-231 cells were transduced with replication-defective retroviruses containing the expression vector pMSCVpuro or MSCVpuro-MSP, and infected cells were selected with puromycin.

4.7.9 Western blot analyses

Cells were serum starved (0.5% FBS) for 3 hours prior to lysate collection. Inhibitors were added 1 hour prior to lysis and MSP was added 15 minutes prior to lysis when indicated. Cells were washed with 1x PBS and harvested in lysis buffer (25mM Tris-HCl, pH 7.5, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.5 mM EDTA, 1 mM DTT, 25% sucrose, and 0.1% Triton X-100) plus 100x protease arrest (Bioscience) and 100x orthovanadate. Primary antibodies used for immunoblotting include: anti-MSP (Santa Cruz, sc-6090) at a 1:250 dilution, anti-pan Akt (Cell Signaling, #4691S) at a 1:1000 dilution, anti-pan Erk (Cell Signaling, #4695) at a 1:1000 dilution, anti-phospho Akt (Cell Signaling, #9271L)

at a 1:500 dilution, anti-phospho Erk (Cell Signaling, #4370S) at a 1:500 dilution, anti-Src (Cell Signaling, #2110) at a 1:500 dilution, and anti-phospho Src (Y416) (Cell Signaling, #2113) at a 1:500 dilution. The secondary antibodies used were HRP-conjugated anti-goat IgG (Santa Cruz) and anti-rabbit IgG (Santa Cruz). Chemiluminescent signals were detected using Immun-Star HRP (BioRad).

4.7.10 *In vivo* bone labeling and histomorphometry

To label bones, mice were intraperitoneally injected with 20 mg/kg of calcein (Sigma-Aldrich) or 30 mg/kg of Alizarin Complexone (Sigma-Aldrich) in a 2% sodium bicarbonate solution. Mice 6 to 8 months of age were labeled 8 days and 2 days prior to euthanasia, respectively. Tibia were fixed in 70% ethanol, embedded in methylmethacrylate, and sectioned. Bone histomorphometry on H and E sections and bone labeled sections were performed as previously described (Parfitt et al., 1987). Analysis of bone volume (BV/TV) and trabecular spacing (Tb. Sp.) were performed on the metaphyseal region of tibia sections stained with H and E. Bone formation rate (BFR) per bone surface (BS) and mineral apposition rate (MAR) of trabeculae were calculated within the metaphyseal region of the tibia. For all histomorphometry, three sections per mouse with 4 to 5 mice per group were analyzed. Analysis was performed using Bioquant Osteo software (Bioquant Image Analysis Corp.).

4.7.11 Statistical Analysis

Data are shown as mean +/- SEM. All experiments were analyzed using 2-tailed, unpaired, Student's t test using Prism 6 (GraphPad Software).

4.8 Acknowledgments

We thank Harold Moses for the kind gift of the PyMT/TGFβRII-/- cells and Bill Dougall (Amgen) for the muRANK-Fc inhibitor. We also would like to thank Scott Miller for help with bone histomorphometry and critical reading of this manuscript. This work was supported by the Department of Defense Breast Cancer Research Program (to A.L.W. – W81XWH0810109 and K.A. – W81XWH1010366), and the Huntsman Cancer Foundation/Institute (to A.L.W.). We also utilized the HCI Comparative Oncology Core and shared resources, which are supported in part by NCI Cancer Center Support Grant P30-CA042014.

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Figure 4.1. MSP increases tumor-driven osteolysis through host Ron activity. (A) Microcomputed tomography (μCT) of bone lesions in the tibia 21 days post tumor cell injection. Scale bar, 2mm. **(B)** Quantification of osteolytic area in tibias from mice *ex vivo* using high resolution X-ray analysis (n=10) 42 days post tumor cell injection. **(C)** Tumor growth in tibias, determined by caliper measurements. **(D)** Proliferation rate of PyMT control and MSP-expressing tumor cells expressed as the percent of cells staining positive for phospho-Histone H3. **(E)** TRAP staining on sections of tumor-bearing bones from each experimental group. Scale bar, 100 μm. **(F)** Quantification of $TRAP^+$ osteoclasts in each experimental group (n=5-7 per group). Data in the figure represent mean +/- SEM; p values were based on Student's t test. ******* $p < 0.0005$, ns = not significant.

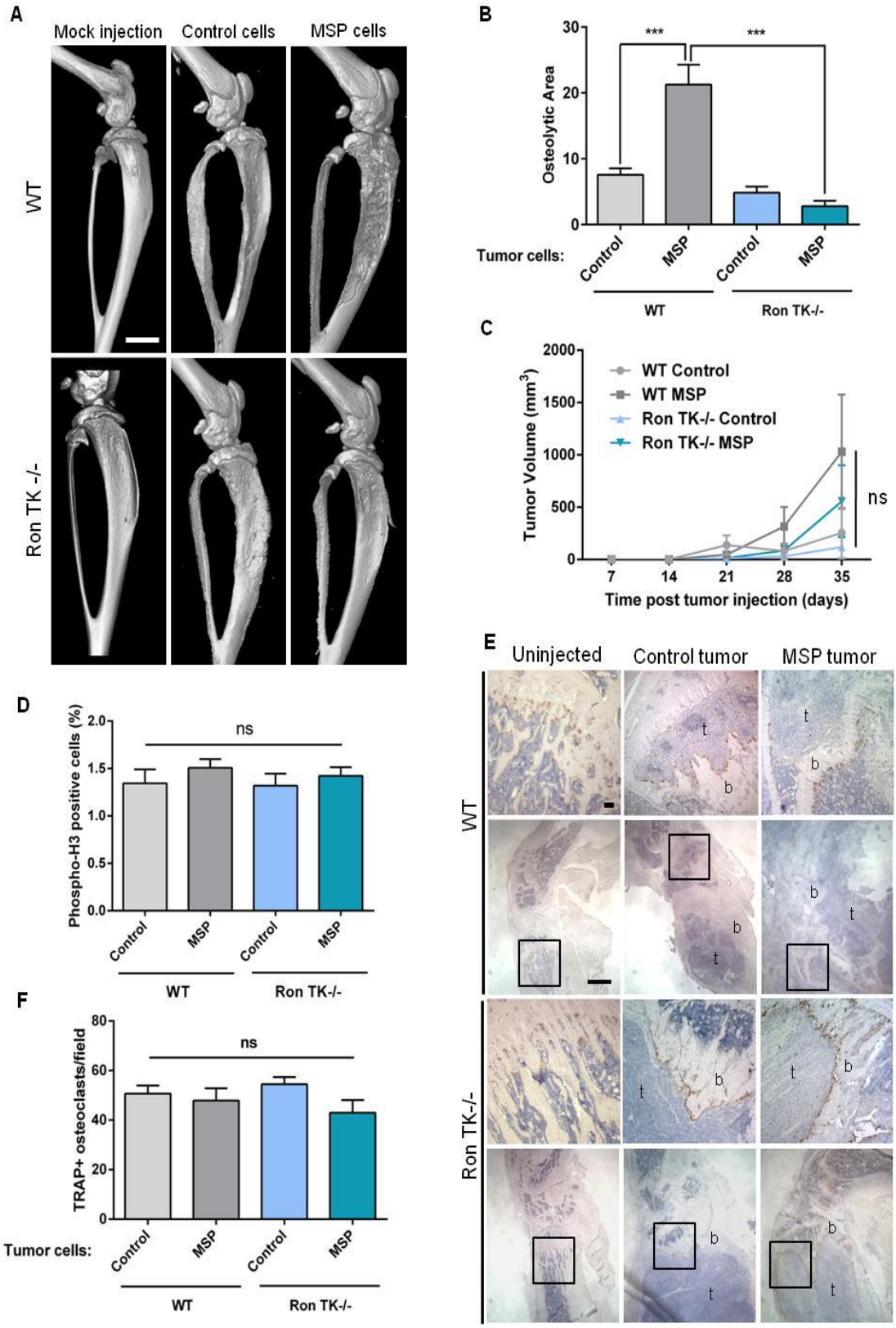
A
Mock injection
Control cells
MSP cells
WT
Ron TK -/-
B
Osteolytic Area
***
***
Tumor cells:
Control
MSP
Control
MSP
WT
Ron TK-/-
C
Tumor Volume (mm3)
WT Control
WT MSP
Ron TK-/- Control
Ron TK-/- MSP
ns
Time post tumor injection (days)
D
Phospho-H3 positive cells (%)
ns
WT
Ron TK-/-
E
Uninjected
Control tumor
MSP tumor
WT
Ron TK-/-
F
TRAP+ osteoclasts/field
ns
Tumor cells:
WT
Ron TK-/-

Figure 4.2. Ron is required for tumor-driven osteolysis of human breast cancer. (A) Representative X-ray images of DU4475 bone lesions in NOD.SCID/ WT or NOD.SCID/Ron TK-/- mice, 42 days post tumor cell injection. **(B)** Quantification of osteolytic area from tibial bone lesions (n=5). **(C)** TRAP staining on sections of tumor-bearing bones from each experimental group. Scale bar, 100 µm. **(D)** Quantification of $TRAP^{+}$ osteoclasts in each experimental group (n=3-4). **(E)** Proliferation rate of DU4475 tumor cells in the bone (n=3-4). ns = not significant. Data in the figure represent mean +/- SEM; p values were based on Student's t test. ***** $p < 0.05$, ns = not significant.

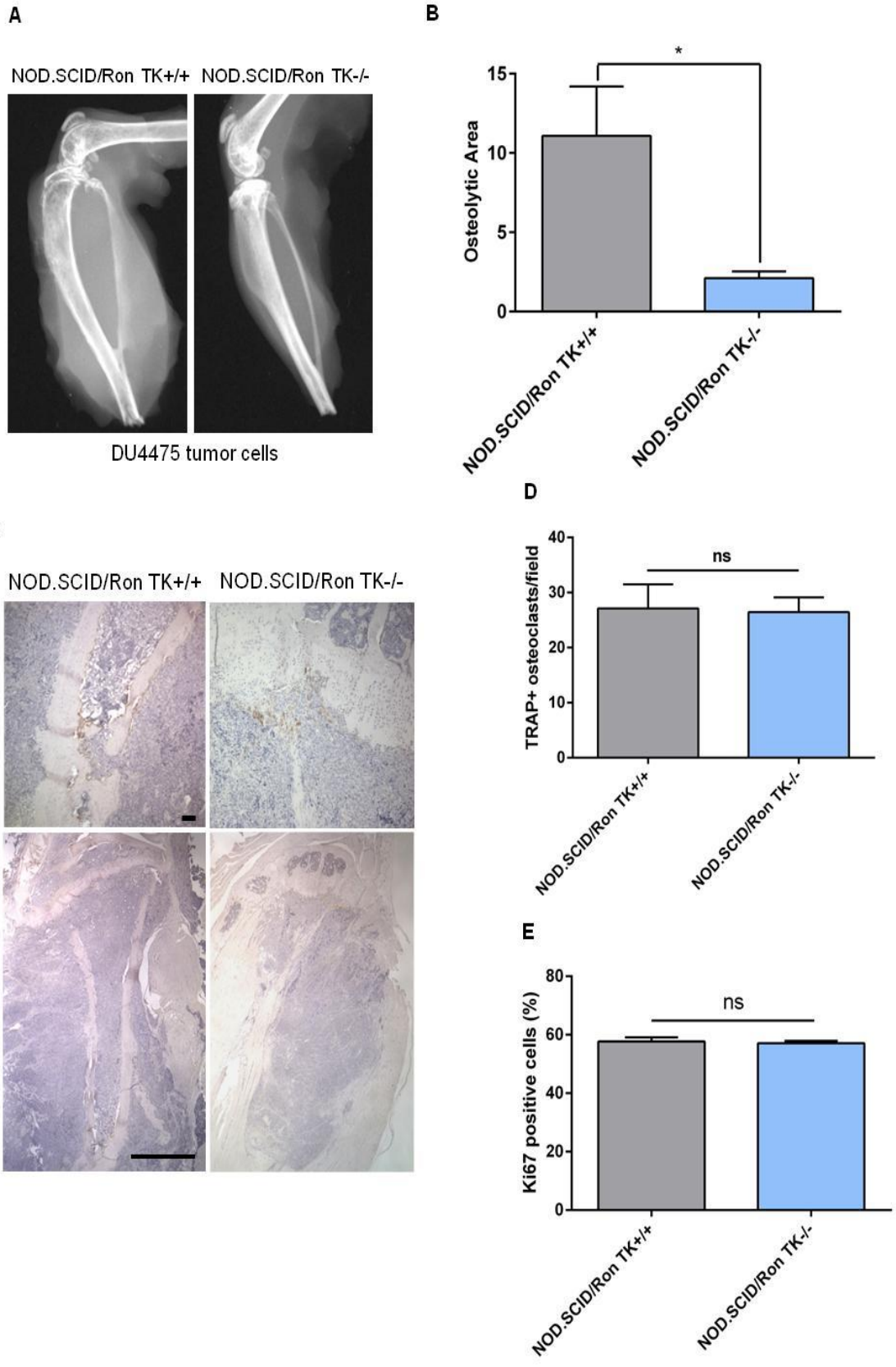
A
NOD.SCID/Ron TK+/+
NOD.SCID/Ron TK-/-
DU4475 tumor cells
B
Osteolytic Area
15
10
5
0
*
NOD.SCID/Ron TK+/+
NOD.SCID/Ron TK-/-
C
NOD.SCID/Ron TK+/+
NOD.SCID/Ron TK-/-
D
TRAP+ osteoclasts/field
40
30
20
10
0
ns
NOD.SCID/Ron TK+/+
NOD.SCID/Ron TK-/-
E
Ki67 positive cells (%)
80
60
40
20
0
ns
NOD.SCID/Ron TK+/+
NOD.SCID/Ron TK-/-

Figure 4.3. Ron inhibition reduces MSP tumor-induced osteolysis in prophylactic and adjuvant settings. (A) X-ray images of PyMT-MSP bone lesions from mice treated with OSI-296. Treatment began 3 days post tumor cell injection for pre-osteolysis treatment and 3 weeks post injection for post osteolysis treatment. Mice were sacrificed 42 days post injection for analysis. **(B)** X-ray images of PyMT-MSP bone lesions from mice treated with ASLAN002. Treatment began 3 days post tumor cell injection. Mice were sacrificed 21 days post injection for analysis. **(C)** Quantification of osteolytic area in PyMT-MSP bone lesions from mice treated with OSI-296 (n=7-10 per group). **(D)** Quantification of osteolytic area in PyMT-MSP bone lesions from mice treated with ASLAN002 (n=4-5 per group). **(E)** Representative X-ray images of DU4475-induced bone lesions from mice treated with ASLAN002. Treatment began 3 days post tumor cell injection and mice were sacrificed 28 days post injection for analysis. **(F)** Quantification of osteolytic area in DU4475 bone lesions from mice in each experimental group (n=5). **(G)** ELISA-based quantification of serum RANKL concentration from mice in each experimental group (n=3). Data in the figure represent mean +/- SEM; p values were based on Student's t test. ***** $p = 0.01$, ****** $p < 0.0074$, ns = not significant.

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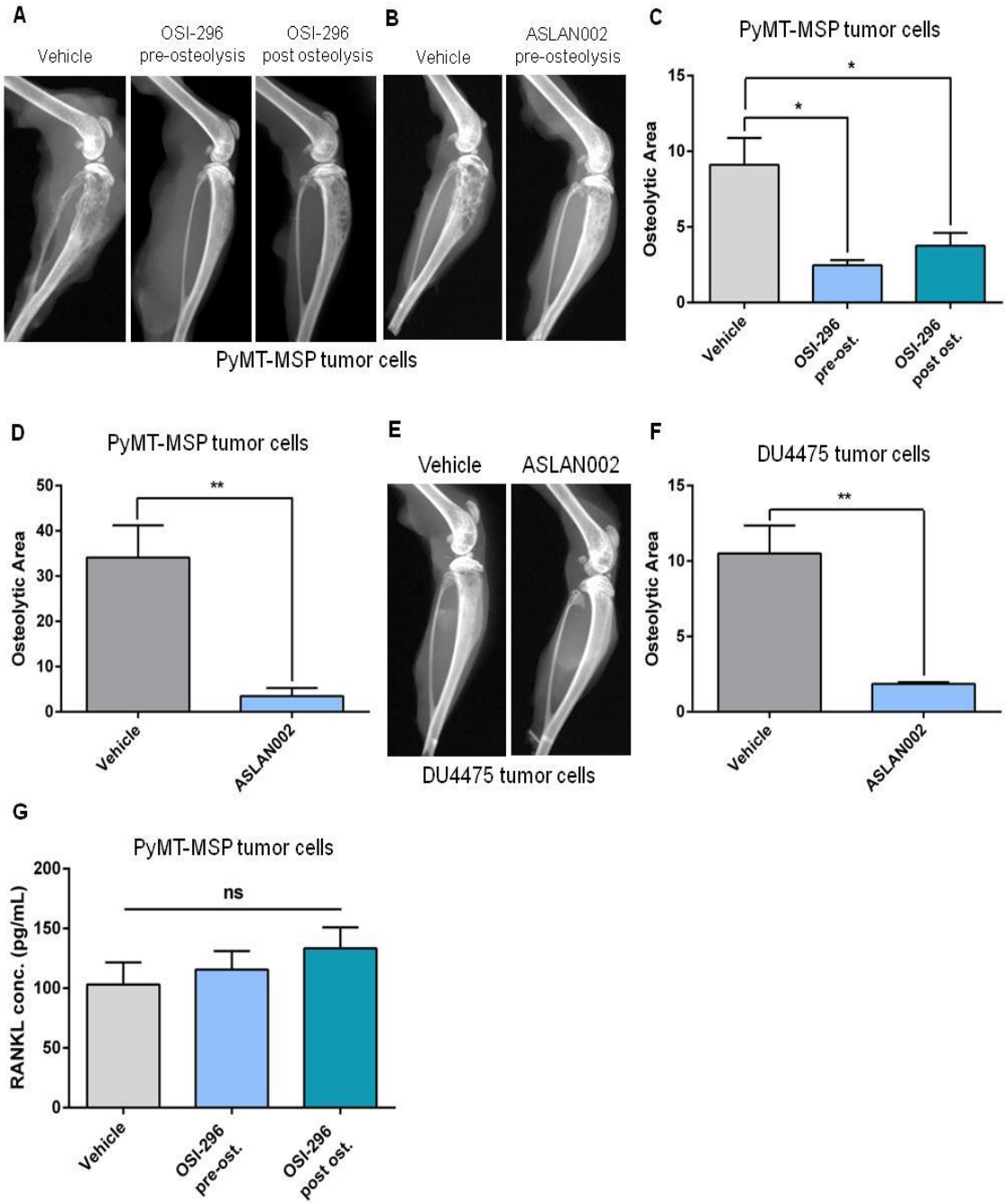

A
Vehicle
OSI-296 pre-osteolysis
OSI-296 post osteolysis
B
Vehicle
ASLAN002 pre-osteolysis
PyMT-MSP tumor cells
C
PyMT-MSP tumor cells
Osteolytic Area
Vehicle
OSI-296 pre-ost.
OSI-296 post ost.
D
PyMT-MSP tumor cells
Osteolytic Area
Vehicle
ASLAN002
E
Vehicle
ASLAN002
DU4475 tumor cells
F
DU4475 tumor cells
Osteolytic Area
Vehicle
ASLAN002
G
PyMT-MSP tumor cells
RANKL conc. (pg/mL)
ns
Vehicle
OSI-296 pre-ost.
OSI-296 post ost.

Figure 4.4. MSP tumor-induced osteolysis is not dependent on RANKL or TGFβ signaling. (A) Representative X-ray images of PyMT-MSP bone lesions from mice treated with muRANK-Fc. Treatment began 3 days post tumor cell injection and mice were sacrificed 42 days post tumor cell injection for analysis. **(B)** Quantification of osteolytic area in bone lesions from mice in each experimental group (n=16 per group for WT mice and 3 to 5 per group for Ron TK-/- mice, respectively). **(C)** TRAP staining of tumor-bearing bones from each experimental group. Scale bar, 100 μm. **(D)** X-ray images of PyMT bone lesions arising from tumors in the presence or absence of TGFβRII. Mice were sacrificed 38 days post tumor cell injection for analysis. **(E)** Quantification of osteolytic area in bone lesions from mice in each experimental group (n=5). Data in the figure represent mean +/- SEM; p values were based on Student's t test. ****** $p = 0.0041$, ******* $p < 0.0001$, ns = not significant.

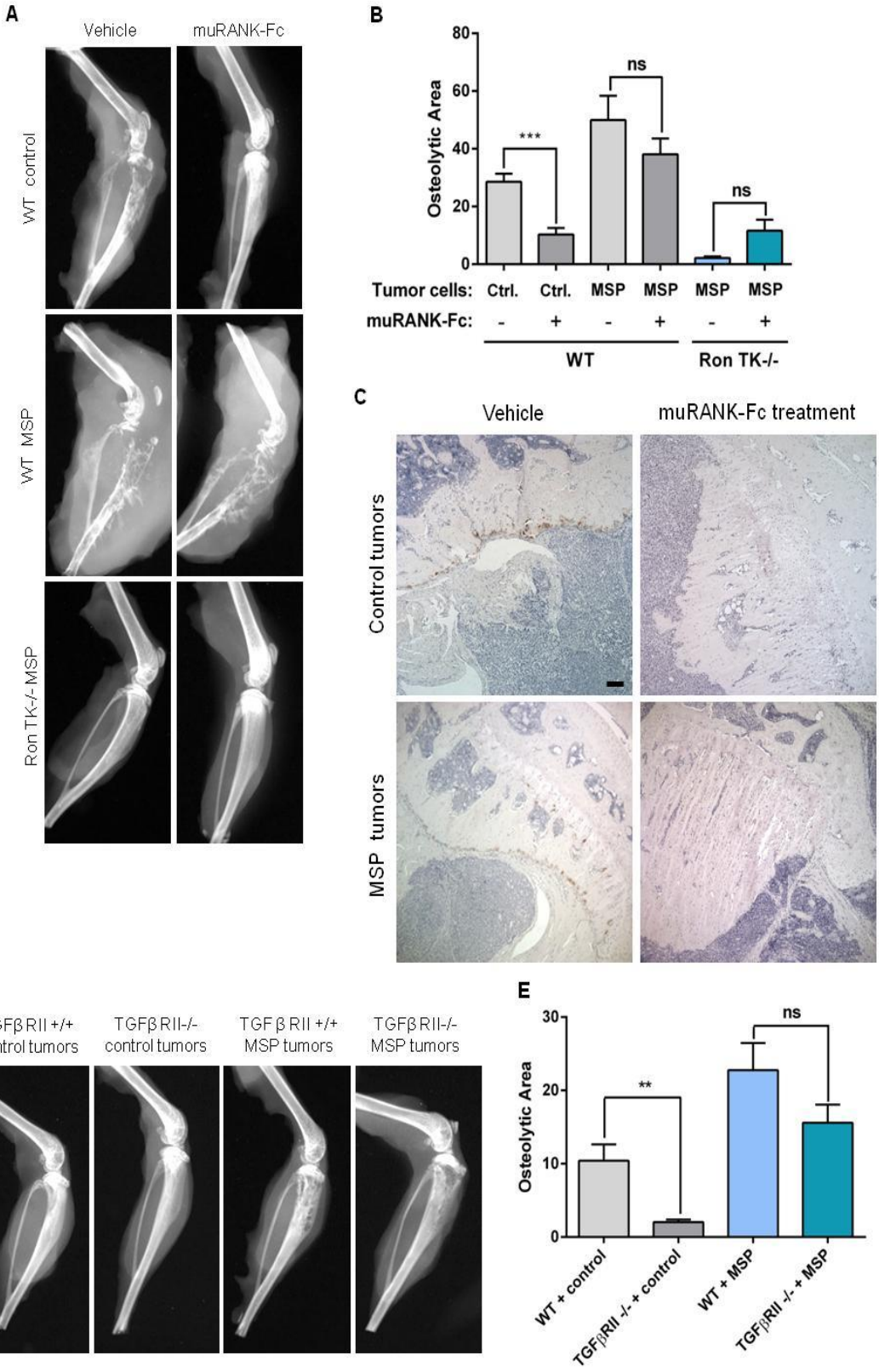

A
Vehicle
muRANK-Fc
WT control
WT MSP
Ron TK-/- MSP
B
Osteolytic Area
80
60
40
20
0
***
ns
ns
Tumor cells: Ctrl. Ctrl. MSP MSP MSP MSP
muRANK-Fc: - + - + - +
WT
Ron TK-/-
C
Vehicle
muRANK-Fc treatment
Control tumors
MSP tumors
D
TGFβ RII +/+ control tumors
TGFβ RII-/- control tumors
TGF β RII +/+ MSP tumors
TGFβ RII-/- MSP tumors
E
Osteolytic Area
30
20
10
0
**
ns
WT + control
TGFβRII -/- + control
WT + MSP
TGFβRII -/- + MSP

Figure 4.5. Expression of MSP in human breast cancer increases tumor-induced osteolysis and overrides dependence on RANKL signaling. (A) Western blot showing MSP protein levels secreted into the media of parental, control, and MSP-overexpressing MDA-MB-231 cells. **(B)** X-ray images of bone lesions from NOD.SCID mice treated with muRANK-Fc or OSI-296 3 days post tumor cell injection. Mice were sacrificed 30 days post tumor cell injection for analysis. **(C)** Quantification of osteolytic area from bone lesions in mice from each experimental group (n=5). Data in the figure represent mean +/- SEM; p values were based on Student's t test. ******p = 0.0014, *******p< 0.0002, ns = not significant.

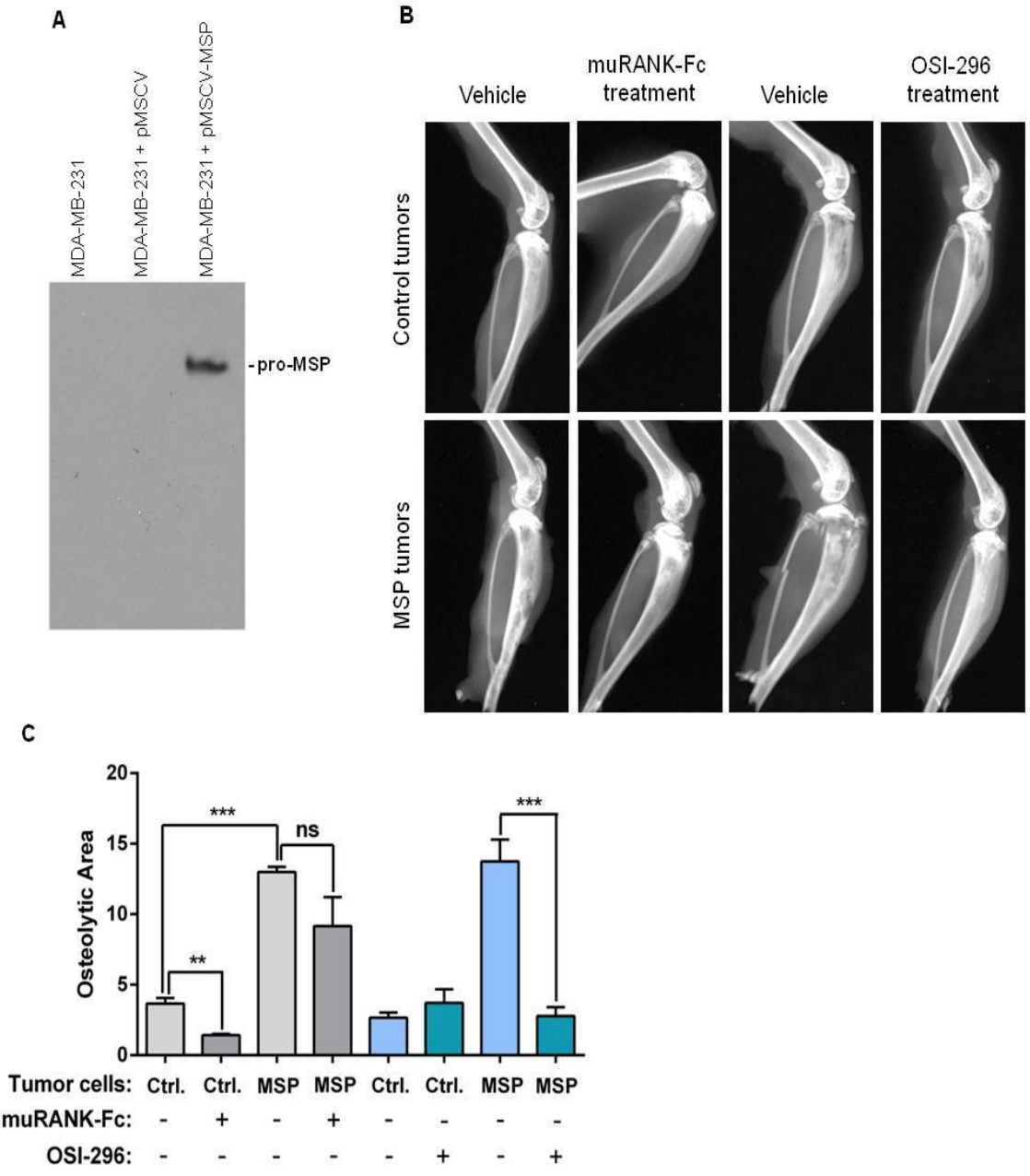

A
MDA-MB-231
MDA-MB-231 + pMSCV
MDA-MB-231 + pMSCV-MSP
- pro-MSP
B
Vehicle
muRANK-Fc treatment
Vehicle
OSI-296 treatment
Control tumors
MSP tumors
C
Osteolytic Area
20
15
10
5
0
***
ns
**
***
Tumor cells: Ctrl. Ctrl. MSP MSP Ctrl. Ctrl. MSP MSP
muRANK-Fc: - + - + - - - -
OSI-296: - - - - - + - +

Figure 4.6. MSP promotes osteoclast activity and survival through activation of Src and Akt signaling pathways. (A) Quantification of the resorption area from each experimental group (n=4). Bone marrow precursor cells were differentiated in the presence of M-CSF and RANKL for 9 days. 100 pg/mL of MSP was added daily beginning on day 9 with the experiment ending on day 12. **(B)** Quantification of the resorption area arising from WT and Ron TK-/- bone marrow precursor cells differentiated in the presence of M-CSF and RANKL for 9 days (n=3). 10 µg/mL of muRANK-Fc was added daily beginning on day 9 with the experiment ending on day 12. **(C)** Quantification of the resorption area (n=3). WT bone marrow precursor cells were seeded on osteologic slides and differentiated in the presence of M-CSF and RANKL for 9 days. muRANK-Fc at a concentration of 10 µg/mL and/or ASLAN002 at a concentration of 10 µM was added daily beginning on day 9 with the experiment ending on day 12. **(D)** Quantification of the number of TRAP+ osteoclasts per well from each experimental group (n=3). WT bone marrow precursor cells were differentiated in the presence of M-CSF and RANKL for 7 days. M-CSF and RANKL were washed out and 100 pg/mL of MSP was added daily, beginning on day 7, and continued for 48 hours. TRAP+ cells were identified by fluorescent TRAP stain and cells with 4 or more nuclei were counted. **(E)** Western blots of osteoclast lysates from WT cells treated with MSP, muRANK-Fc, and /or ASLAN002. **(F)** Model of MSP/Ron function in the bone microenvironment. Data in the figure represent mean +/- SEM; p values were based on Student's t test. *****$p < 0.04$, ******$p < 0.01$, *******$p < 0.0001$, ns = not significant.

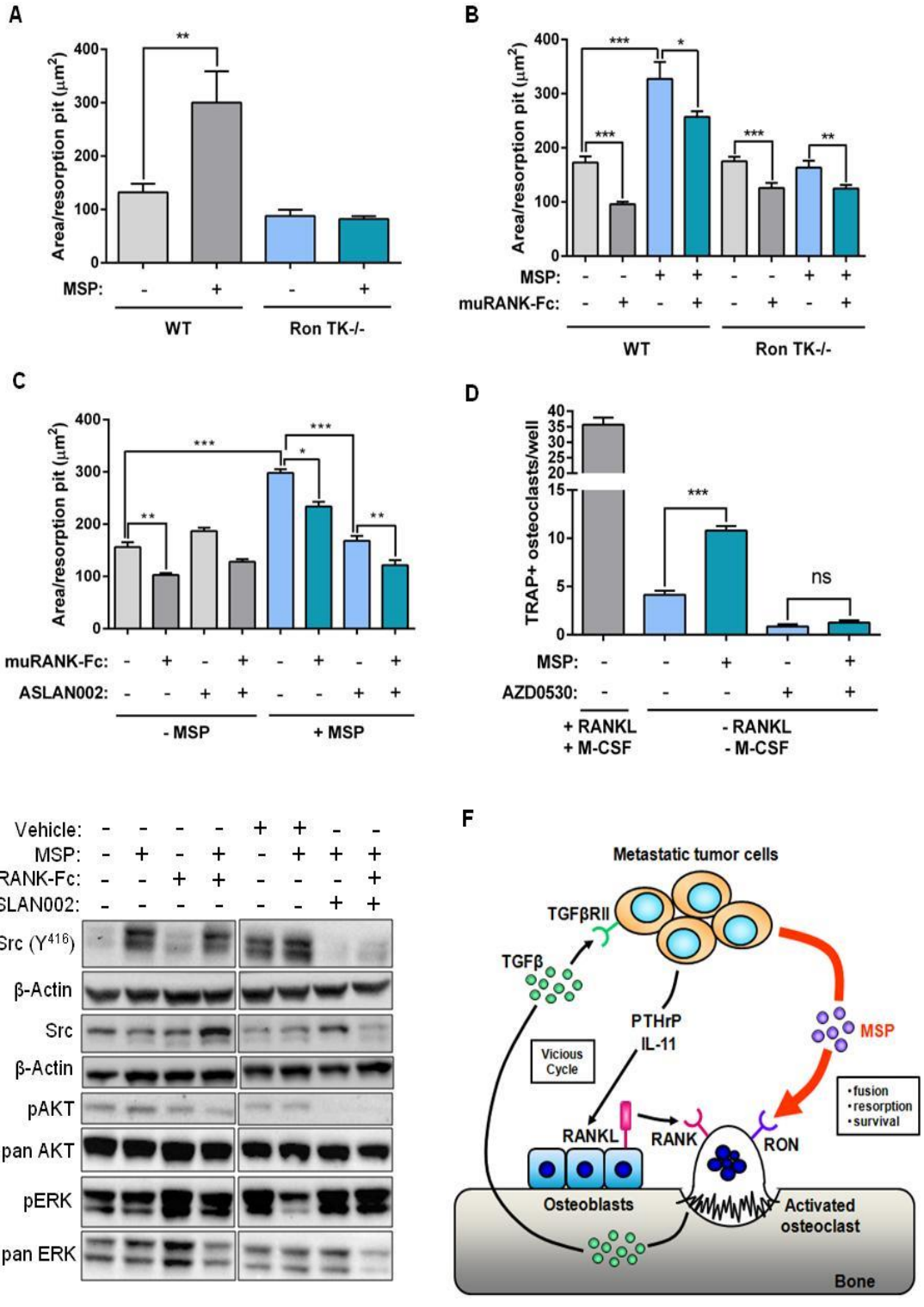

A
Area/resorption pit (μm²)
MSP:
WT
Ron TK-/-
B
Area/resorption pit (μm²)
MSP:
muRANK-Fc:
WT
Ron TK-/-
C
Area/resorption pit (μm²)
muRANK-Fc:
ASLAN002:
- MSP
+ MSP
D
TRAP+ osteoclasts/well
ns
MSP:
AZD0530:
+ RANKL
+ M-CSF
- RANKL
- M-CSF
E
Vehicle:
MSP:
muRANK-Fc:
ASLAN002:
pSrc (Y416)
β-Actin
Src
β-Actin
pAKT
pan AKT
pERK
pan ERK
F
Metastatic tumor cells
TGFβRII
TGFβ
PTHrP
IL-11
Vicious Cycle
MSP
•fusion
•resorption
•survival
RANKL
RANK
RON
Osteoblasts
Activated osteoclast
Bone

Figure 4.7. Loss of Ron activity protects from osteoporotic bone loss. (A) μCT images of the proximal tibia (axial view of the metaphyseal region) from WT and Ron TK-/- mice following ovariectomy (OVX) or sham operation. WT OVX mice were treated with ASLAN002 beginning 1 day postovariectomy. Mice were sacrificed 28 days postovariectomy for analysis. **(B)** Quantification of bone mineral density in the metaphyseal region of the tibia determined by bone histomorphometry analysis (n=5). **(C)** Quantification of trabecular bone volume in the tibia expressed at percent per total volume and determined by bone histomorphometry analysis (n=5). **(D)** Quantification of trabecular separation in the tibia (n=5). **(E)** Representative sections of the tibia from each group stained for H and E. Data in the figure represent mean +/- SEM; p values were based on Student's t test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ns = not significant.

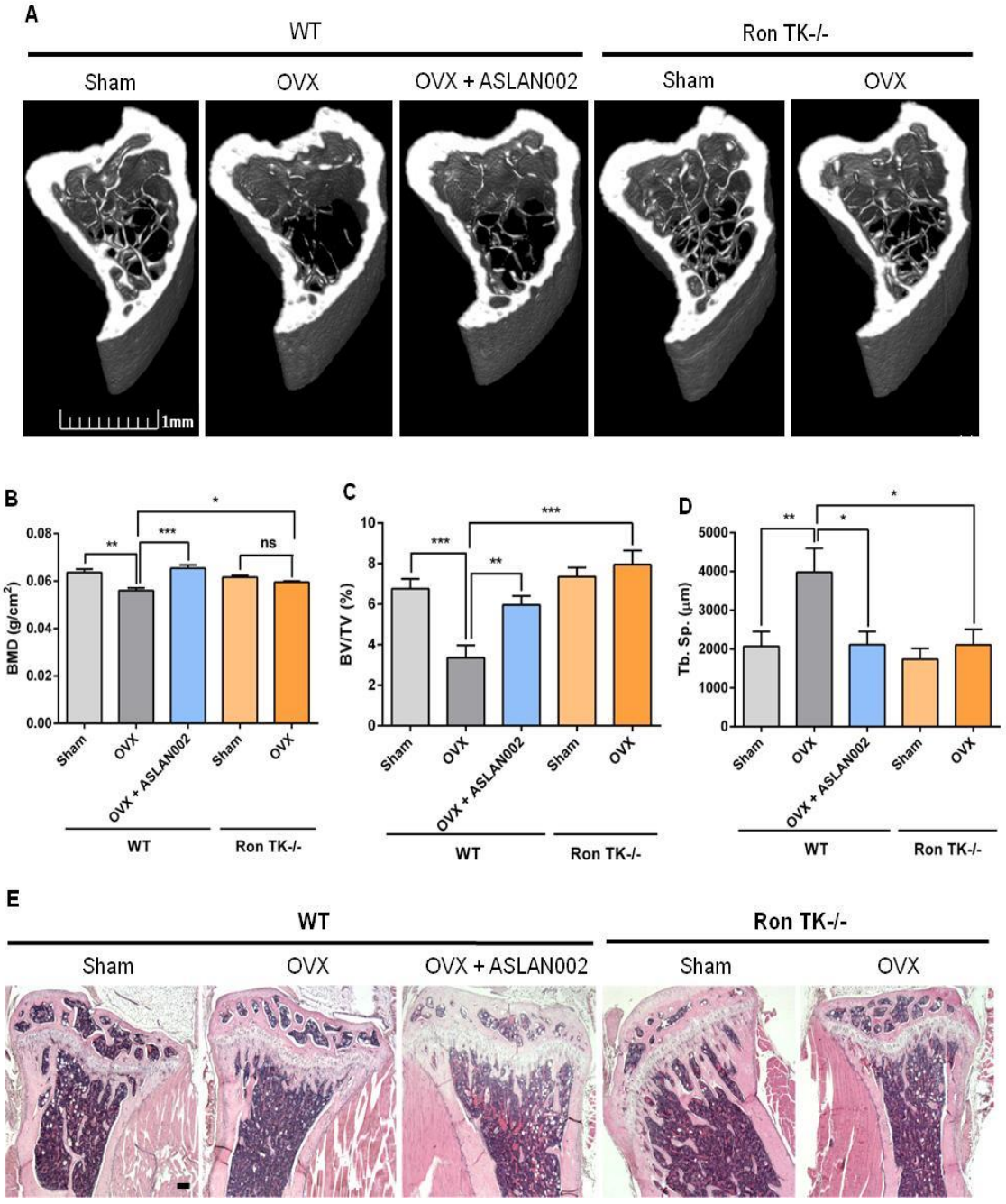

A
WT
Ron TK-/-
Sham
OVX
OVX + ASLAN002
Sham
OVX
1mm
B
BMD (g/cm²)
0.08
0.06
0.04
0.02
0.00
**
***
*
ns
Sham
OVX
OVX + ASLAN002
Sham
OVX
WT
Ron TK-/-
C
BV/TV (%)
10
8
6
4
2
0
***
***
**
Sham
OVX
OVX + ASLAN002
Sham
OVX
WT
Ron TK-/-
D
Tb. Sp. (μm)
5000
4000
3000
2000
1000
0
**
*
*
Sham
OVX
OVX + ASLAN002
Sham
OVX
WT
Ron TK-/-
E
WT
Ron TK-/-
Sham
OVX
OVX + ASLAN002
Sham
OVX

Figure 4S1. Genetic deletion of Ron has no effect on osteoblast activity (A) Quantification of TRAP+ osteoclasts from normal, uninjected WT and Ron TK-/- mice (n=5). **(B)** Bone formation as observed by calcein and Alizarin double labeling, scale bar = 100 µm for images at 4x magnification and 50 µm for inset images at 40x magnification. **(C)** Mineral apposition rate determined by bone histomorphometric analysis in WT and RonTK-/- mice (n=4-5). **(D)** Bone formation rate per total bone surface in WT and RonTK-/- mice (n=4-5). **(E)** Mineral apposition rate in WT and STK-/- mice (n=4-5). **(F)** Bone formation rate per total bone surface in WT and STK-/- mice (n=4-5). **(G)** Tissue sections stained for Ron in normal, uninjected WT and STK -/- mice. Both osteoclasts and chondrocytes stain positive for Ron. **(H)** Western analysis for Ron and phospho-Ron in the MCF10A-Ron and MC3T3-E1 cell lines. Cells were grown in the presence of 10% serum to 90% confluency. The media was then exchanged for media containing 0.5% serum and the cells were cultured overnight. 100 pg/mL MSP was added for 15 minutes and lysates were prepared. The MCF10A cell line overexpressing Ron serves as the positive control for Ron and phospho-Ron expression while the MC3T3-E1 cell line demonstrates the lack of Ron expression in osteoblasts. ns = not significant.

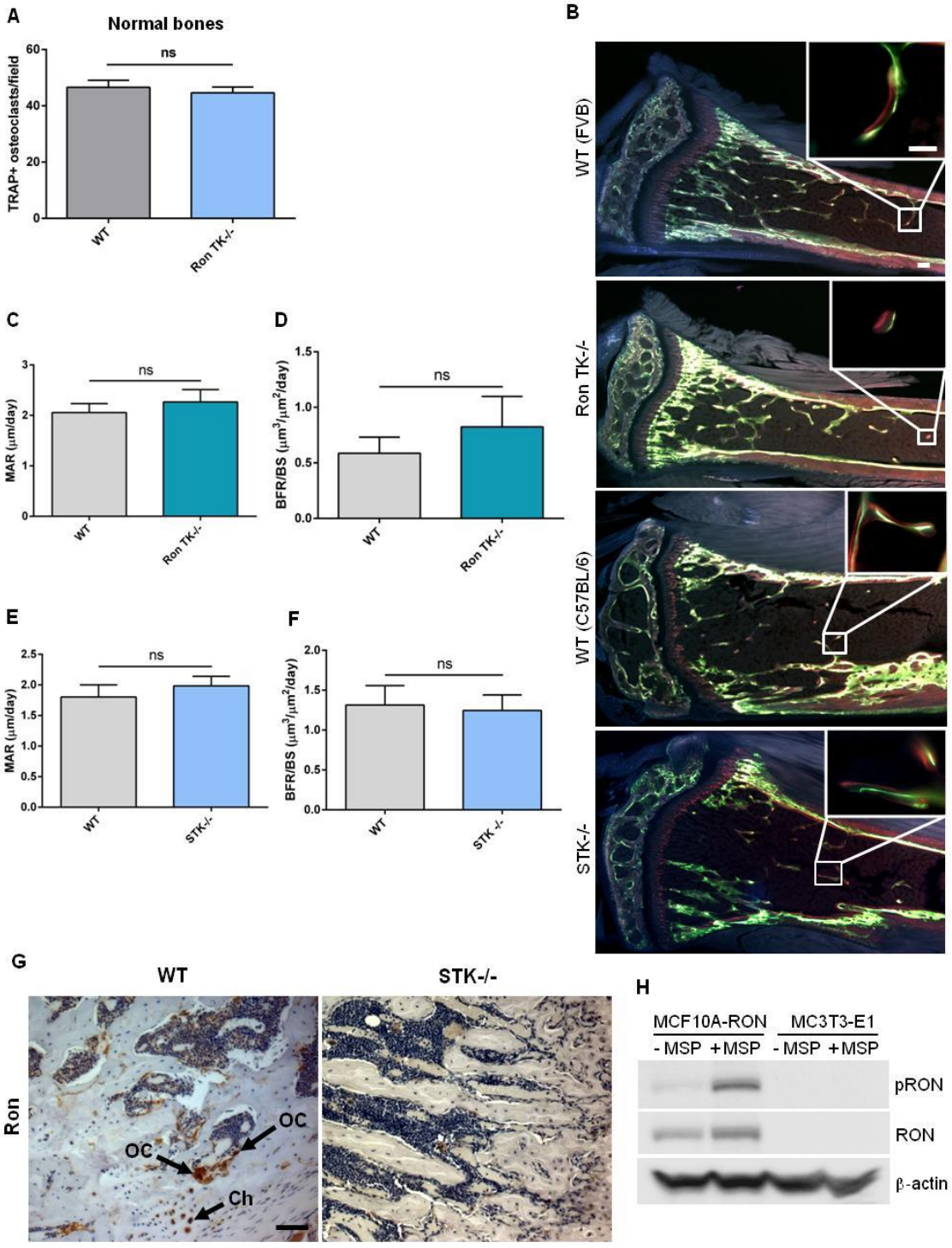

A
Normal bones
TRAP+ osteoclasts/field
ns
WT
Ron TK-/-
B
WT (FVB)
Ron TK-/-
WT (C57BL/6)
STK-/-
C
MAR (μm/day)
ns
WT
Ron TK-/-
D
BFR/BS (μm³/μm²/day)
ns
WT
Ron TK-/-
E
MAR (μm/day)
ns
WT
STK-/-
F
BFR/BS (μm³/μm²/day)
ns
WT
STK -/-
G
WT
STK-/-
Ron
OC
OC
Ch
H
MCF10A-RON
MC3T3-E1
- MSP
+MSP
- MSP
+MSP
pRON
RON
β-actin

Figure 4S2. MSP-driven osteolysis does not depend on T cells (A) mRNA expression levels of MSP, Ron, and matriptase in a panel of human breast cancer cell lines. Dotted lines depict average expression level for each gene. **(B)** TRAP staining of normal, uninjected NOD.SCID:WT and NOD.SCID:Ron TK-/- bones. Scale bar, 100 µm. **(C)** Quantification of TRAP+ osteoclasts from normal, uninjected bones (n=4). **(D)** X-ray images of bone lesions from each group. PyMT tumor cells expressing MSP were injected into the tibia of NOD.SCID: WT or NOD.SCID:Ron TK-/- mice. Mice were sacrificed 28 days post tumor cell injection for analysis. **(E)** Quantification of osteolytic area of bone lesions in mice from each experimental group (n=3-5). **(F)** Growth curves of PyMT-MSP bone lesions for each experimental group. Tumor size was determined by caliper measurements. **(G)** TRAP staining of bone tumors from each experimental group. Scale bar, 100 µm. **(H)** Quantification of TRAP+ osteoclasts from PyMT-MSP bone tumors of each experimental group (n=4). Data in the figure represent mean +/- SEM; p values were based on Student's t test. *$p < 0.05$, ns = not significant.

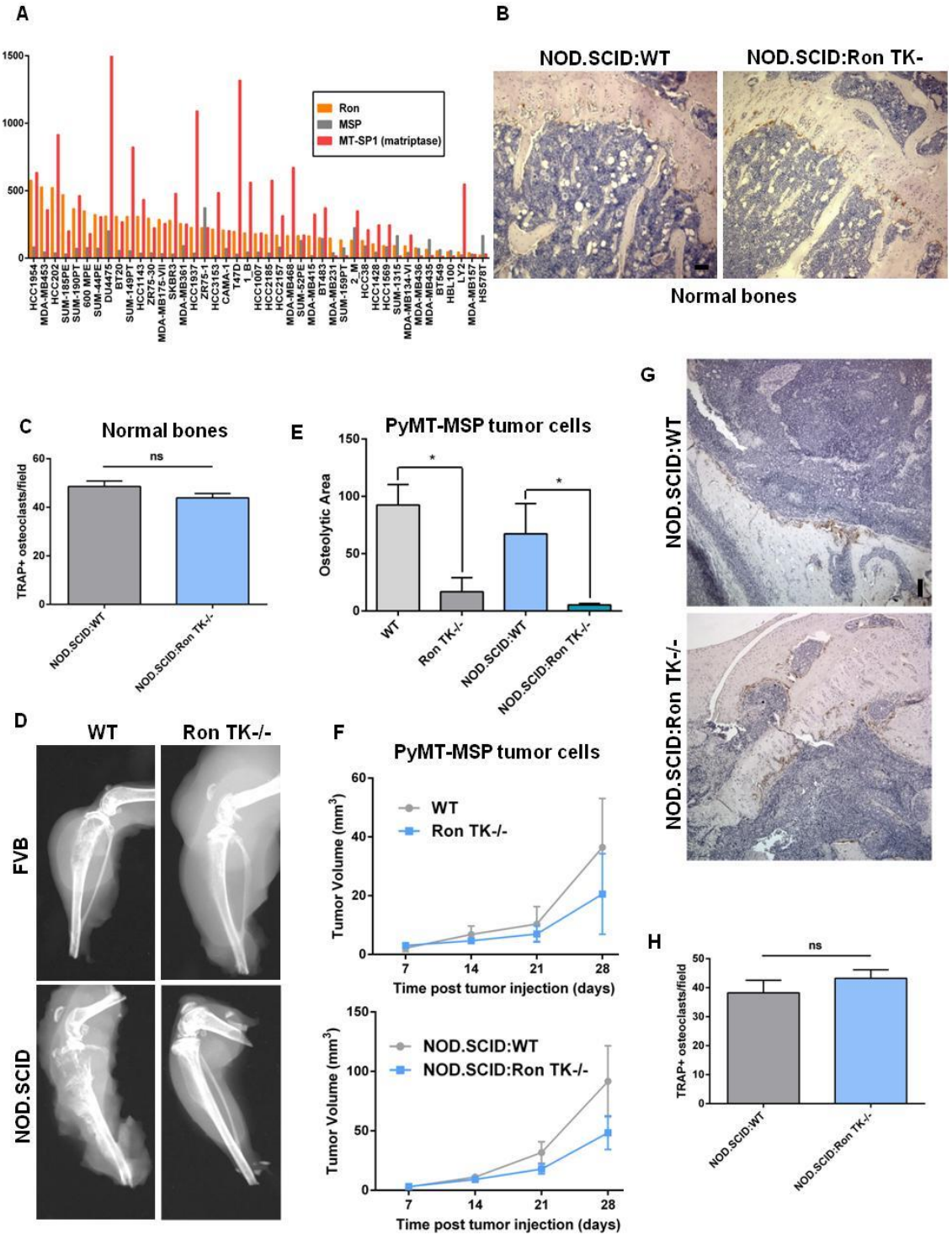

A
Ron
MSP
MT-SP1 (matriptase)
B
NOD.SCID:WT
NOD.SCID:Ron TK-
Normal bones
C
Normal bones
TRAP+ osteoclasts/field
ns
NOD.SCID:WT
NOD.SCID:Ron TK-/-
D
WT
Ron TK-/-
FVB
NOD.SCID
E
PyMT-MSP tumor cells
Osteolytic Area
WT
Ron TK-/-
NOD.SCID:WT
NOD.SCID:Ron TK-/-
F
PyMT-MSP tumor cells
Tumor Volume (mm3)
WT
Ron TK-/-
Time post tumor injection (days)
NOD.SCID:WT
NOD.SCID:Ron TK-/-
G
NOD.SCID:WT
NOD.SCID:Ron TK-/-
H
TRAP+ osteoclasts/field
ns
NOD.SCID:WT
NOD.SCID:Ron TK-/-

Figure 4S3. Ron inhibitors do not significantly reduce tumor growth (A) X-ray images of PyMT control bone lesions treated with OSI-296. **(B)** Quantification of osteolytic area in mice with bone lesions treated with OSI 296 (n=8,5). Control tumor cells were injected into the tibia of WT mice, treatment began 3 days post tumor cell injection. Mice were sacrificed 42 days post injection for analysis. **(C)** Proliferation rate for PyMT control tumors indicated as percent cells staining positive for phospho-H3 (n=3). **(D)** Tumor growth curve (n = 9 - 13) and proliferation rate (n = 5,4) for PyMT-MSP bone lesions treated with OSI-296. PyMT-MSP tumor cells were injected into the tibia of WT mice and treatment began 3 days post tumor cell injection for preosteolysis treatment and 3 weeks post injection for postosteolysis treatment. Mice were sacrificed 42 days post injection for analysis. **(E)** Tumor growth curve and proliferation rate for PyMT-MSP bone lesions treated with ASLAN002 (n=5,4). PyMT-MSP tumor cells were injected into the tibia of WT mice and treatment began 3 days post tumor cell injection. Mice were sacrificed 21 days post tumor cell injection; tumor size was determined by caliper measurements ($n > 0.28$). **(F)** Proliferation rate of PyMT-MSP tumors treated with OSI-296 (n=3). **(G)** Tumor growth curve and proliferation rate for DU4475 bone lesions from each experimental group treated with ASLAN002 (n=5). DU4475 tumor cells were injected into the tibia of NOD.SCID/ Ron TK+/+. Treatment began 3 days post tumor cell injection and mice were sacrificed 42 days post tumor cell injection for analysis ($n > 0.44$). Data in the figure represent mean +/- SEM; p values were based on Student's t test. $*p < 0.02$, ns = not significant.

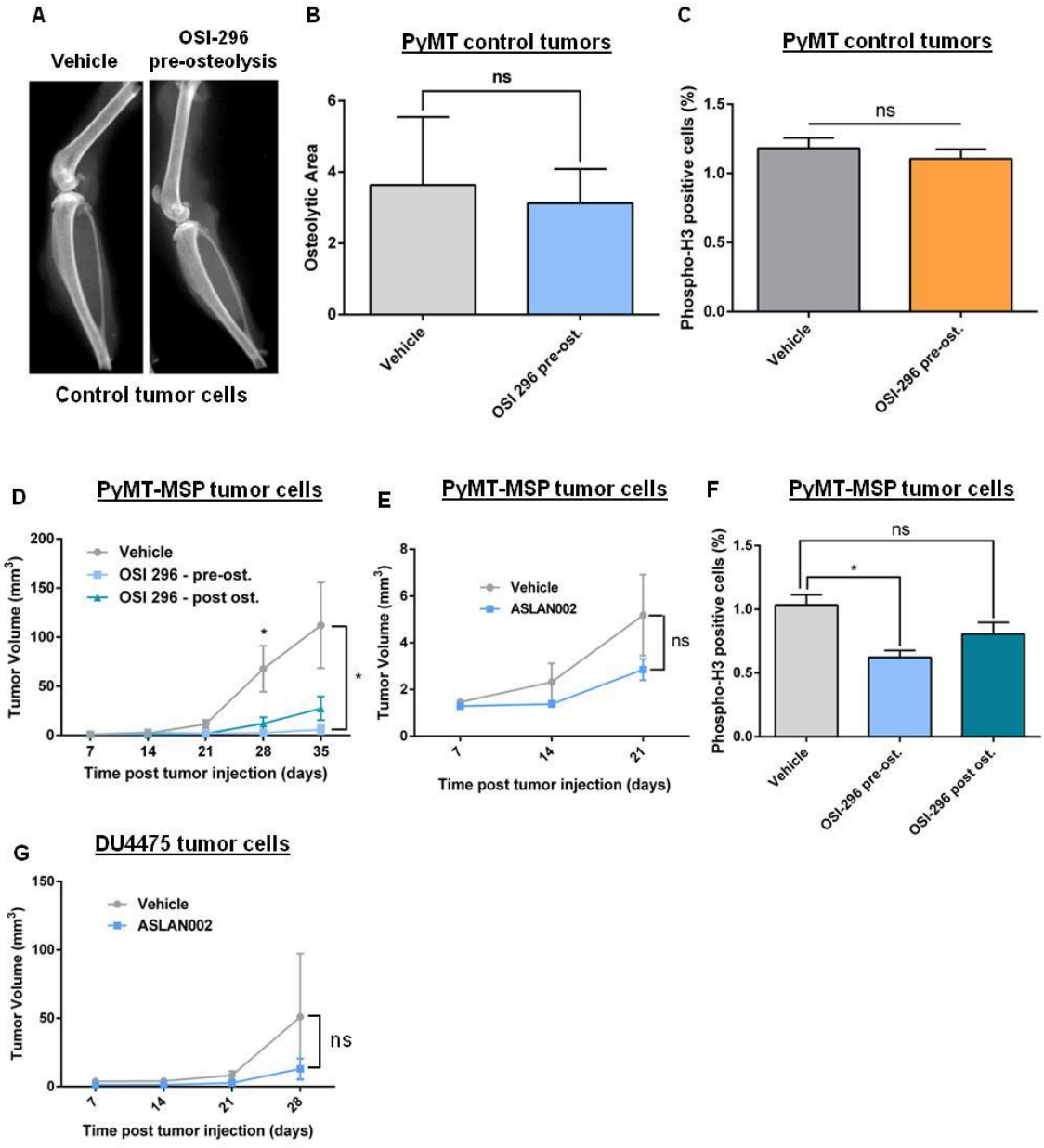

A
Vehicle
OSI-296 pre-osteolysis
Control tumor cells
B
PyMT control tumors
ns
Osteolytic Area
Vehicle
OSI 296 pre-ost.
C
PyMT control tumors
ns
Phospho-H3 positive cells (%)
Vehicle
OSI-296 pre-ost.
D
PyMT-MSP tumor cells
Vehicle
OSI 296 - pre-ost.
OSI 296 - post ost.
Tumor Volume (mm3)
Time post tumor injection (days)
E
PyMT-MSP tumor cells
Vehicle
ASLAN002
ns
Tumor Volume (mm3)
Time post tumor injection (days)
F
PyMT-MSP tumor cells
ns
*
Phospho-H3 positive cells (%)
Vehicle
OSI-296 pre-ost.
OSI-296 post ost.
G
DU4475 tumor cells
Vehicle
ASLAN002
ns
Tumor Volume (mm3)
Time post tumor injection (days)

Figure 4S4. Ron inhibitors do not affect osteoclast number or osteoblast activity (A) TRAP staining of PyMT-MSP bone tumors from mice treated with OSI-296 either 3 days post tumor cell injection for preosteolysis or 3 weeks post injection for postosteolysis treatment. Mice were sacrificed 42 days post injection for analysis. **(B)** Quantification of TRAP+ osteoclasts from bone tumors in each experimental group (n=4). **(C)** Bone formation as observed by calcein and Alizarin double labeling, scale bar, 100 µm for images at 4x magnification, and 50 µm for inset images at 40x magnification. **(D)** Mineral apposition rate in normal, uninjected animals treated with ASLAN002 (n=4 - 5). **(E)** Bone formation rate per total bone surface in animals treated with ASLAN002 (n=4 - 5). Data in the figure represent mean +/- SEM; p values were based on Student's t test. ns = not significant.

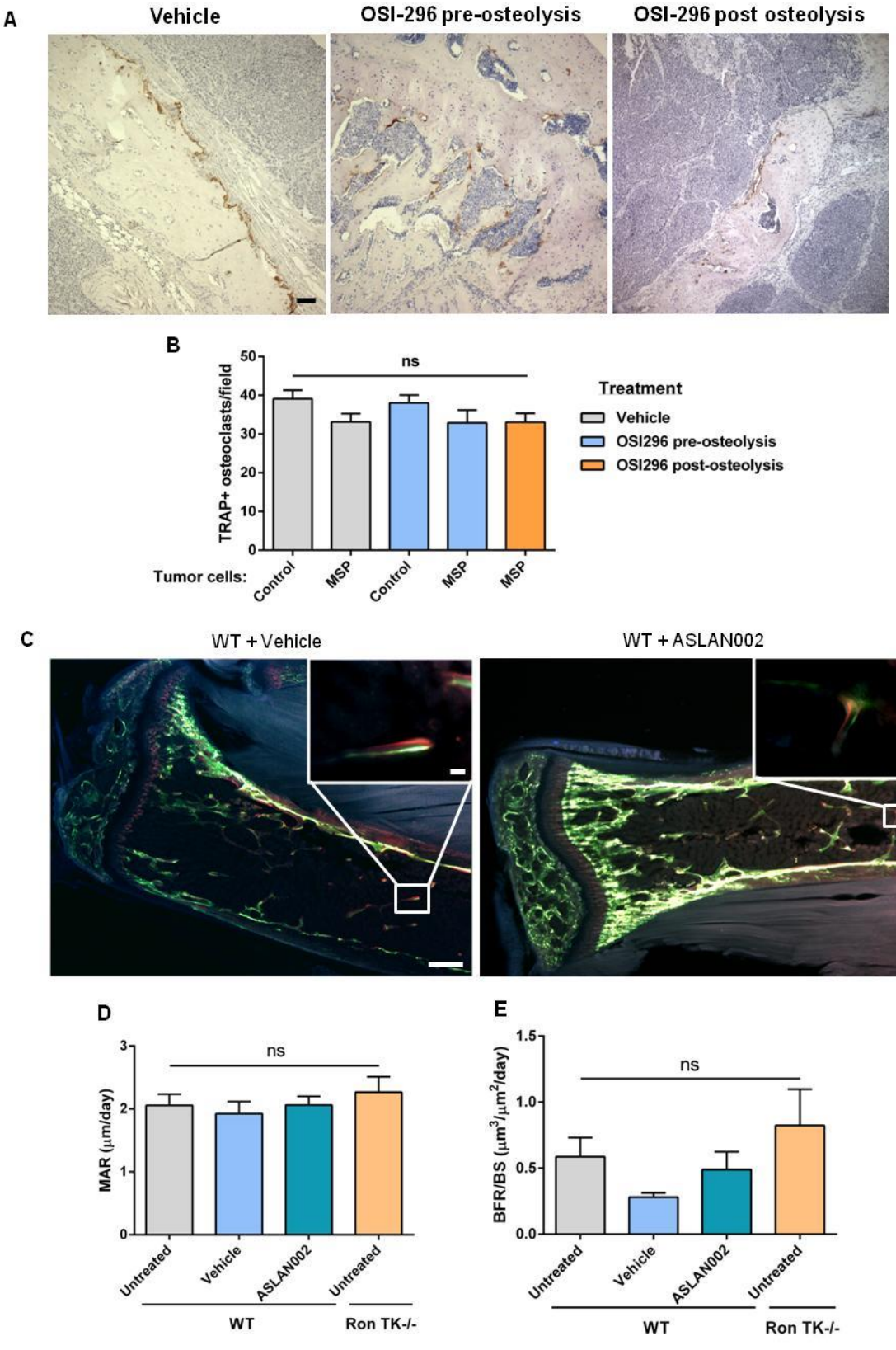

A
Vehicle
OSI-296 pre-osteolysis
OSI-296 post osteolysis
B
TRAP+ osteoclasts/field
ns
Treatment
Vehicle
OSI296 pre-osteolysis
OSI296 post-osteolysis
Tumor cells:
Control
MSP
Control
MSP
MSP
C
WT + Vehicle
WT + ASLAN002
D
MAR (μm/day)
ns
Untreated
Vehicle
ASLAN002
Untreated
WT
Ron TK-/-
E
BFR/BS (μm³/μm²/day)
ns
Untreated
Vehicle
ASLAN002
Untreated
WT
Ron TK-/-

Figure 4S5. Neither RANKL antagonism nor the lack of TGFβ significantly affects tumor growth (A) Growth curve from bone lesions treated with muRANK-Fc in each experimental group. Tumor cells were injected into the tibia of WT or Ron TK-/- mice. Treatment began 3 days post tumor cell injection. Tumor size was determined by caliper measurements. **(B)** Ron and NFATc1 staining of tissue sections from PyMT-MSP tumor-bearing bones treated with muRANK-Fc. Arrows indicate positively stained osteoclasts, scale bar = 50 μm. **(C)** Growth curve of either PyMT wildtype or TGFβRII-/- bone lesions in mice from each experimental group. Tumor cells were injected into the tibia of WT mice. Tumor size was determined by caliper measurements. *p = 0.03, ns = not significant.

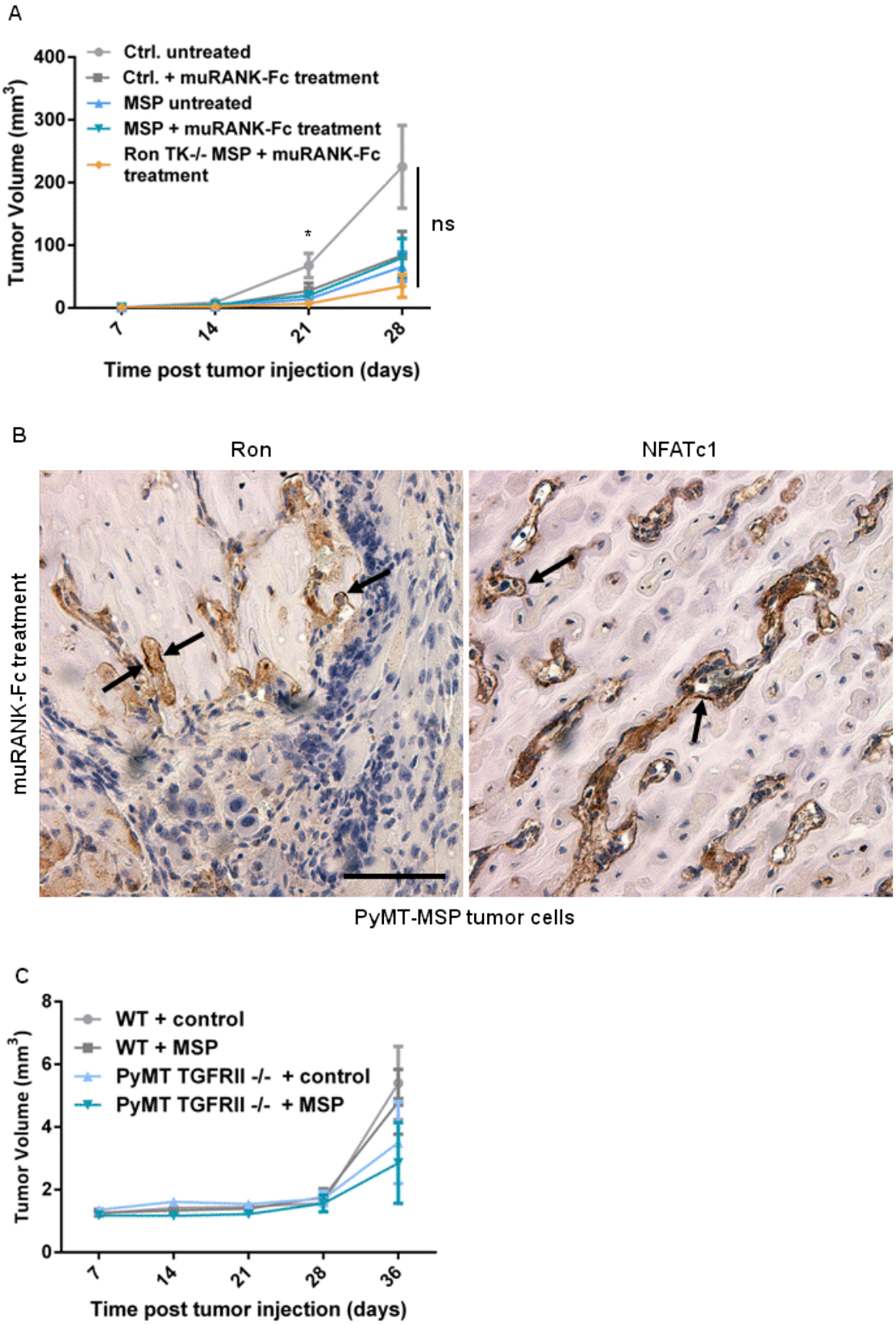
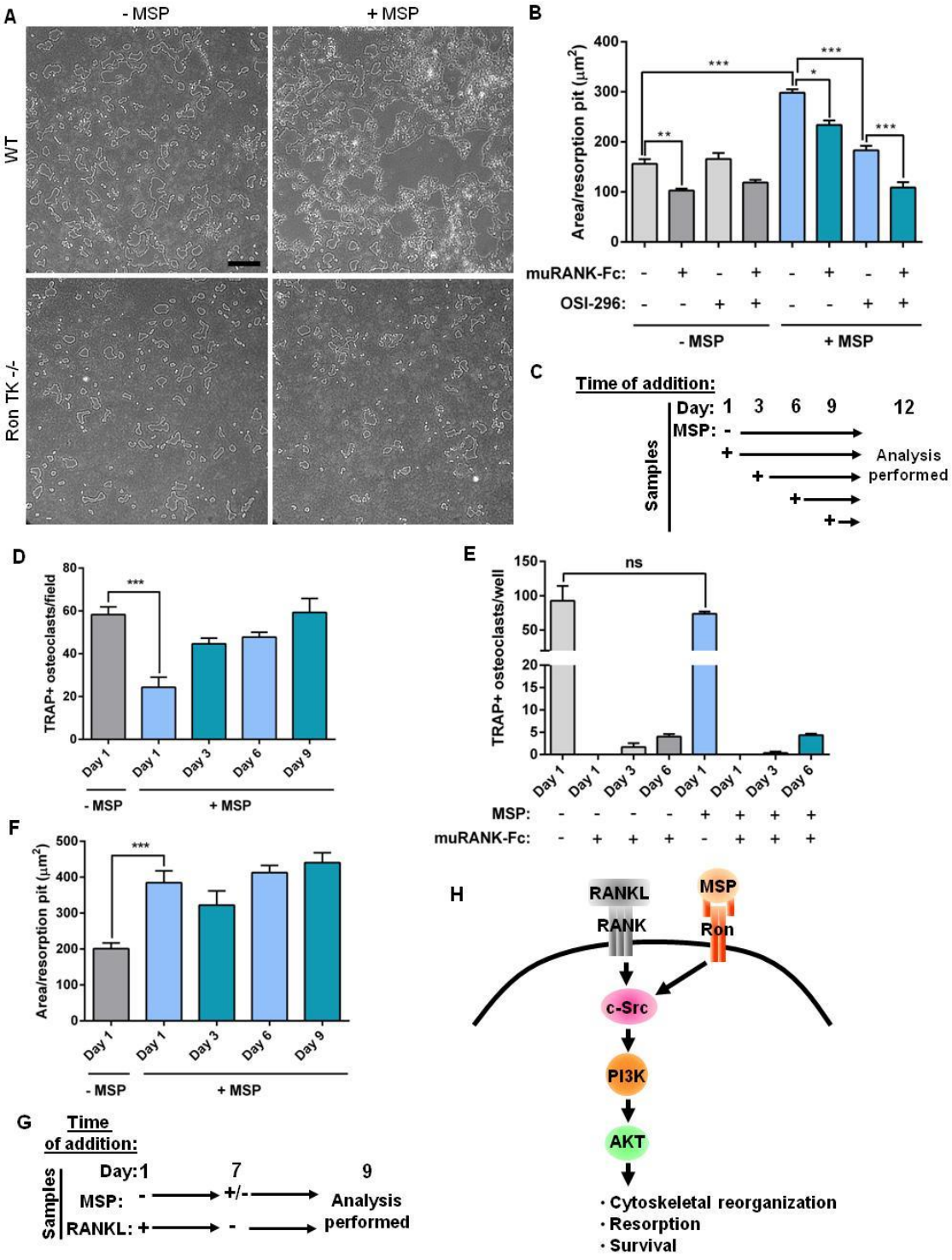

A
Ctrl. untreated
Ctrl. + muRANK-Fc treatment
MSP untreated
MSP + muRANK-Fc treatment
Ron TK-/- MSP + muRANK-Fc treatment
Tumor Volume (mm3)
400
300
200
100
0
*
ns
7
14
21
28
Time post tumor injection (days)
B
Ron
NFATc1
muRANK-Fc treatment
PyMT-MSP tumor cells
C
WT + control
WT + MSP
PyMT TGFRII -/- + control
PyMT TGFRII -/- + MSP
Tumor Volume (mm3)
8
6
4
2
0
7
14
21
28
36
Time post tumor injection (days)

Figure 4S6. MSP increases osteoclast activity independently of RANKL while having no effect on osteoclast differentiation (A) Representative images of resorption pits from WT and Ron TK-/- osteoclasts in the presence and absence of MSP. **(B)** Quantification of resorption area in each experimental group (n=3). WT bone marrow precursor cells were seeded on osteologic slides and differentiated in the presence of M-CSF and RANKL for 9 days. muRANK-Fc at a concentration of 10 µg/mL and/or OSI-296 at a concentration of 10 µM was added daily beginning on day 9, ending on day 12. **(C)** Timeline describing the addition of MSP during differentiation. M-CSF and RANKL were present continuously beginning on day 1 until experimental analysis. **(D)** Quantification of TRAP+ osteoclasts in each experimental group (n=3). WT bone marrow precursor cells were seeded on glass coverslips and differentiated in the presence of M-CSF and RANKL. 100 pg/mL of recombinant MSP was added daily, beginning at the time points indicated. TRAP+ cells were identified by fluorescent TRAP stain and cells with 4 or more nuclei were counted. **(E)** Quantification of the number of TRAP+ osteoclasts per well for each experimental group (n=3). WT bone marrow precursor cells were seeded onto glass coverslips and differentiated in the presence of M-CSF and RANKL. RANKL was removed from the media and 100 pg/mL of recombinant MSP was added at the indicated time points with the experiment terminating on day 9. **(F)** Quantification of resorption area in each experimental group (n=3). WT bone marrow precursor cells were seeded on osteologic slides and differentiated in the presence of M-CSF and RANKL for 9 days. 100pg/mL of recombinant MSP was added at the indicated time points with the experiment terminating on day 12. **(G)** Timeline describing the addition of MSP and removal of RANKL to determine osteoclast survival. Cells were cultured in the presence of M-CSF and RANKL for 7 days. These factors were then removed from the culture and 100 pg/mL of MSP was added daily, beginning on day 7, and continued for 48 hours. **(H)** Model of signaling pathways activated downstream of Ron activation in osteoclasts. Data in the figure represent mean +/- SEM; p values were based on Student's t test. *$p < 0.03$, **$p < 0.002$, ***$p < 0.0001$.


A
- MSP
+ MSP
WT
Ron TK -/-
B
Area/resorption pit (µm²)
muRANK-Fc:
OSI-296:
- MSP
+ MSP
C
Time of addition:
Day: 1 3 6 9 12
MSP:
Samples
Analysis performed
D
TRAP+ osteoclasts/field
Day 1
Day 1
Day 3
Day 6
Day 9
- MSP
+ MSP
E
TRAP+ osteoclasts/well
ns
MSP:
muRANK-Fc:
F
Area/resorption pit (µm²)
G
Time of addition:
Day:1 7 9
MSP:
RANKL:
Samples
Analysis performed
H
RANKL
RANK
MSP
Ron
c-Src
PI3K
AKT
· Cytoskeletal reorganization
· Resorption
· Survival

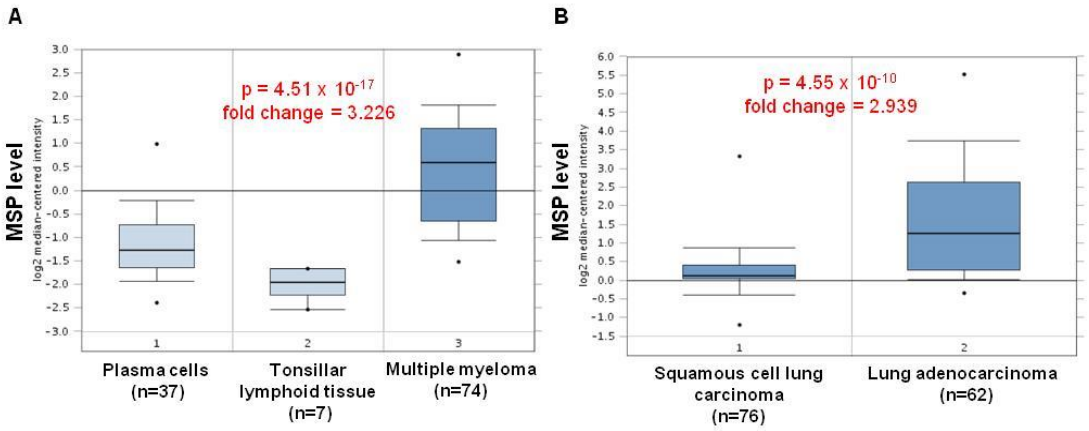


Figure 4S7. MSP is highly expressed in other bone-metastatic cancers when compared to normal cell types (A) Expression of MSP in normal plasma cells versus myeloma cells. **(B)** Expression of MSP in lung carcinomas.

CHAPTER 5

DISCUSSION

5.1 Ron function in osteoclasts

Stem cell-derived tyrosine kinase (STK) is the murine homologue of the human RON gene and was originally isolated from mouse hematopoietic stem cells (Ronsin 1993). STK protein was also shown to be expressed on resident peritoneal macrophages, suggesting a role for STK in multiple cell types from the monocytic lineage (Iwama 1995). Based on this evidence, Kurihara et al. hypothesized that STK may be expressed on osteoclasts as well, given that they are derived from similar precursor cells. STK was found to be expressed on osteoclasts differentiated with IL-3 and vitamin D3. The addition of MSP to these cells to *in vitro* cultures induced contraction and formation of the ruffled border, increased resorption, and caused redistribution of Src to the ruffled border (Kurihara 1996). Ron was then shown to be expressed on osteoclasts cultured from human bone marrow cells. As was demonstrated in murine osteoclasts, addition of MSP to human osteoclasts resulted in a cytoplasmic contraction, redistribution of Src to the ruffled border, and osteoclast resorption

In addition, MSP was shown to have no effect on osteoclast differentiation or DNA synthesis in osteoclast progenitor cells, in contrast to functions of HGF (Kurihara 1998).

My data agree with the original observations that MSP has no critical role in osteoclast differentiation but has a marked effect on osteoclast resorption (Figures 4.S6D and 4.S6F). I have significantly extended these findings by showing that the lack of, or inhibition of, Ron kinase activity results in a significant reduction in resorption activity. I also showed that MSP can prolong survival of osteoclasts in the absence the key osteoclast cytokines M-CSF and RANKL. Importantly, I have shown that the ability of MSP to induce both osteoclast activity and survival is independent of RANKL signaling but requires c-Src activity. These results demonstrate that, at least in part, activation of Ron on osteoclasts by MSP results in downstream Src signaling.

c-Src has been implicated in several key osteoclast functions including cytoskeletal reorganization, podosome assembly, and osteoclast survival. Src-/- mice have normal osteoclast numbers but develop severe osteopetrosis due to inactivity of these osteoclasts (Soriano 1990). Given the importance of c-Src in osteoclast activation, the ability of Ron to augment c-Src activity could explain how Ron functions to stimulate osteoclast activity. c-Src is known to signal downstream of RANK by associating with TRAF6. The ability of Ron to activate Src presents an explanation for osteoclast activity that is independent of RANKL, with Ron functioning as an alternative activator of c-Src.

The mechanisms by which Ron promotes osteoclast survival and activity are still under investigation, but plausible hypotheses can be gleaned from our knowledge of Ron function in other cell types. Ron signaling in various normal cells is classically mediated by RAS-ERK and PI3K-AKT pathways. For signaling activation, the interaction of Ron with adaptor proteins such as growth factor receptor-bound protein 2 (GRB2) is the first step in bridging Ron with downstream signaling cascades (Chaudhuri 2011; Li 1995). Various cytoplasmic effector molecules such as phospholipase C γ (PLCγ), PI3K, Src, 14-3-3, and Cbl physically interact with Ron through the C-terminal docking site (Yao 2013). During carcinogenesis, Ron has also been shown to activate NF-kB and signal transducer and activator of transcription (STAT) pathways (Park 2011; Xu 2005). In keratinocytes, Ron has been shown to regulate integrins via 14-3-3 proteins, which then regulate adhesion and migration of these cells. Stimulation of keratinocytes with MSP leads to phosphorylation of both Ron and α6β4 integrin and the formation of 14-3-3 binding sites within these proteins. Dimeric 14-3-3 proteins then mediate the relocation of α6β4 integrins, concomitant activation of α3β1 integrin, and its relocalization from the plasma membrane to focal contacts over the migrating front, thereby culminating in cell spreading and migration (Santoro 2003). Interestingly, the Ron kinase does not directly activate α6β4, suggesting that another kinase, possibly a Src-like kinase, may be involved. The mechanism regulating activation of α3β1 in this context is unclear; however, the proposed model involves Ron signaling and 14-3-3/α3β1 complexes being responsible for a shift in integrin dominance, thereby allowing α3β1 to move to

focal contacts and regulate actin-myosin driven processes (Santoro 2003). The mechanisms underlying the ability of integrins to transition from a mechanical adhesion complex to signaling complexes are unclear; however, this evidence suggests that Ron is capable of regulating adhesion and migration through the regulation of integrins.

Several integrins are involved in osteoclast binding to bone matrix proteins, including αvβ3 (binds to osteopontin, vitronectin, and bone sialoprotein), αvβ5 (binds to fibronectin), and α2β1 (binds to collagen) (Schneider 2011). Of these, αvβ3 has been shown to be the predominant integrin found on osteoclasts, and inhibition of αvβ3 blocks osteoclast attachment to the bone matrix, as well as osteoclast-mediated bone resorption (Ross 1993). αvβ3 signaling has also been shown to be critical for creation of the resorptive ruffled membrane, regulation of osteoclast spreading, and overall organization of the cytoskeleton. αvβ3 has also been shown to be important for activation of c-Src, c-Cbl, and the GTPases Rho and Rac, all of which are necessary for the cytoskeletal reorganization important to osteoclast function (Schneider 2011).

Because Ron has the ability to activate and regulate integrins in other cell types, such as keratinocytes, it is possible that Ron functions to regulate integrins in osteoclasts as well. Activation of integrins could explain the ability of MSP/Ron to significantly increase osteoclast resorption. This may also explain why addition of MSP to osteoclasts is able to cause relocalization of c-Src to the ruffled border and cytoplasmic contraction. RANK has been shown to interact with αvβ3 via c-Src to promote actin reorganization in the osteoclast (Izawa 2012). It is possible

that Ron regulates osteoclast function independently of RANK but via activation of similar, critical pathways. Finally, Ron has been shown to promote survival of various cancer cells through MAPK and PI3K signaling (Danilkovitch 2000). The activation of PI3K leads to activation of Akt, which has been shown to enhance cell survival in osteoclasts, suggesting the possibility of a similar mechanism for Ron regulating survival in osteoclasts (Wong 1999).

While I have clearly demonstrated the ability of Ron to activate c-Src in osteoclasts, much more work is needed to determine the signaling pathways downstream of Ron in osteoclasts and the functions they regulate. Determining the pathways involved will also allow for the discovery of transcription factors regulated by MSP/Ron to regulate osteoclast gene expression, furthering our understanding of osteoclast biology and how MSP/Ron signaling culminates in a transcriptional response. It is likely that regulation of PI3K/Akt by Ron functions in osteoclast survival but this still needs to be determined. Utilizing docking domain mutants of Ron that alter downstream signaling could help determine the pathways involved in specific functions within osteoclasts (Iwama 1996; Ponzetto 1994), especially if used in a rescue setting within the Ron TK-/- osteoclasts.

In addition to the need for a better understanding of signaling pathways in regulation of osteoclast activity, the osteoclast itself (and structures important to its function) needs to be analyzed in much greater detail. Determining whether MSP has an effect on formation of the ruffled border, organization of podosomes and/or the assembly of actin-associated proteins will aid in determining potential mechanisms by which Ron regulates osteoclast resorption. While actin ring

assembly appears to be generally normal in Ron TK-/- osteoclasts, this needs to be analyzed utilizing more technical microscopy with live imaging and antibodies specific to actin-associated proteins to determine whether there are any perturbations in actin assembly or the recruitment of these proteins. Analysis of acid production and vesicle formation may also give clues as to why osteoclasts are much more active upon stimulation by MSP.

5.2 Potential for Ron inhibitors in clinical trials

Patients with bone metastases develop skeletal-related events (SREs) defined as a pathologic fracture, requirement for surgical intervention or palliative radiotherapy, hypercalcemia, and/or spinal cord compression. SREs are associated with significant loss of mobility, pain, and reduced quality of life, and negatively affect survival (Coleman 2001). New therapies that prevent and/or delay SREs, reduce bone pain, and improve quality of life are much needed.

Currently, bisphosphonates are the established standard of care for patients with bone metastases. Bisphosphonates are potent inhibitors of osteoclast-mediated bone resorption through several mechanisms, including induction of osteoclast apoptosis and reduction of osteoclast activity (Petrut 2008). The clinical efficacy of bisphosphonates primarily stems from two key properties: their ability to bind strongly to bone mineral, and their inhibitory effects on mature osteoclasts (Russell 2008). Their strong attachment to bone gives bisphosphonates the unique property of selective uptake by their intended target (osteoclasts). The adsorption of bisphosphonates to hydroxyapatite bone mineral

surfaces brings them into close contact with osteoclasts and other cells in the bone. During bone resorption, the acidic environment created by the osteoclast in the resorption lacuna is thought to allow the dissociation of bisphosphonates from the hydroxyapatite (Ebetino 1998). The bisphosphonates are then taken up by osteoclasts within intracellular endocytic vesicles. The bisphosphonates used as standard of care currently, such as zoledronic acid, contain nitrogen in the side chain of their chemical structure. These nitrogen-containing bisphosphonates act principally by inhibiting farnesyl pyrophosphate synthase, thereby preventing the posttranslational modification (prenylation) of small guanosine triphosphate-binding proteins that are essential for osteoclast function and survival (Russell 2008; Baron 2011).

Treatment with zoledronic acid was shown to reduce the risk of developing a SRE by 41%, in addition to reducing bone pain. Treatment also reduces biomarkers of bone turnover such as urinary amino-terminal cross-linked telopeptides of type I collagen (uNTx) and bone-specific alkaline phosphatase (BSAP) by 68% and 37%, respectively (Petrut 2008; Stopeck 2010). Despite its efficacy, zoledronic acid is not without side effects. SREs still occur in a large proportion of patients despite therapy (Rosen 2001; Rosen 2003). Osteonecrosis of the jaw is a rare condition that occurs in patients treated with bisphosphonates, particularly with use of nitrogen-containing bisphosphonates. Nephrotoxicity has been shown to be associated with bisphosphonate therapy and the risk increases with extended treatment, so careful monitoring of creatinine clearance levels is required and termination of therapy may ensue

(Chang 2003; Perazella 2008). Additionally, acute-phase reactions (flu-like symptoms) to intravenous bisphosphonate infusions occur frequently and require treatment with analgesics and antipyretics (Stopeck 2010).

The identification of the RANK/RANKL pathway, and the realization of its importance in osteoclasts, opened up the possibility for new targeted agents for treatment of osteolysis. Denosumab (Xgeva or Prolia), a fully human monoclonal antibody targeting RANKL, was developed and is now FDA approved. Denosumab prevents RANKL from binding to RANK and, in doing so, inhibits the development, activation, and survival of osteoclasts (Baron 2011). A phase III study comparing denosumab with zoledronic acid in patients with breast cancer bone metastases was performed, using the delay or prevention of SREs as an endpoint. Denosumab reduced the risk of developing multiple SREs by 23% and delayed the time to first on-study SRE by 18% compared with zoledronic acid. In addition, denosumab reduced the levels of bone turnover markers: uNTx was reduced by 80% and BSAP was reduced by 44% (Stopeck 2010). A meta-analysis on three separate phase III trials involving breast cancer, prostate cancer, and multiple myeloma demonstrated the superiority of denosumab as compared to zoledronic acid in reducing the risk of the first on-study SRE by 17% (Lipton 2012). While denosumab does appear to be more efficacious than zoledronic acid, it is also much more costly and has a significantly higher risk of hypercalcemia in patients with low renal clearance (Qi 2013). Osteonecrosis of the jaw is still a concern, though the incidence in patients receiving denosumab is similar to the incidence observed with bisphosphonates (Baron 2011). Despite

this advance in treatment, denosumab does not completely block the bone destruction caused by bone metastases and, more importantly, neither bisphosphonates nor denosumab affects disease progression or overall survival, underlining the fact that improved therapies are still needed.

Currently, there is one clinical trial open and one trial completed studying Ron inhibitors in cancer patients. The recently completed trial, NCT01119456, tested the safety and dosing of IMC-RON8 (namatumab) in patients with advanced solid tumors. IMC-RON8 is a monoclonal antibody that prevents the binding of MSP to Ron. While the results of the trial are not yet available, preclinical studies showed that IMC-RON8 blocked MSP-dependent Ron phosphorylation and downstream signaling in pancreatic cancer cell lines, inhibited MSP-driven cell migration, and sensitized these cells to treatment with the HDAC inhibitor panobinostat (Zou 2013). The second trial, NCT01721148, is a phase I trial studying the effects of ASLAN002 (BMS777607) in advanced or metastatic patients with solid tumors. ASLAN002, one of the drugs I used in my studies, is a small molecule tyrosine kinase inhibitor primarily targeting Ron and Met (Schroeder 2009). This inhibitor was originally developed as a Met inhibitor and has been shown in preclinical studies to inhibit a metastatic phenotype in prostate cancer cell lines and a mouse model of sarcoma (Dai 2010; Dai 2012). Despite the focus on Met as the target for this inhibitor, it is about twice as efficacious against Ron (IC50 of 1.8 nM for Ron vs. 3.9 nM for Met) (Schroeder 2009). Indeed, I have demonstrated that this drug successfully inhibited MSP-dependent tumor osteolysis in several models of breast cancer and prevented

bone loss due to osteoporosis (Chapter 4). Because the results obtained using ASLAN002 were phenocopied in experiments performed using Ron knockout mouse models, I believe that my results with ASLAN002 are primarily the result of Ron inhibition. The next step in utilizing our knowledge of Ron biology for therapy is to translate my research results to clinical trials (see Appendix). However, there are many interesting questions that remain regarding the biology of Ron in various diseases, results of which may aid in design of other types of trials.

5.3 Future preclinical studies of MSP/Ron function

While my data have convincingly demonstrated Ron as a potential therapeutic target and ASLAN002 as an efficacious and relatively non-toxic drug in mouse models of breast cancer, there are still questions remaining. Because tumors overexpressing MSP were more osteolytic in mouse models than non-MSP tumors, it is possible that the expression level of MSP in patient tumors may correlate with their bone destructive ability. These data also suggest a potential need for patient selection, or at least stratification, based on MSP expression levels. Currently, there are no standardized techniques to measure or stain for MSP protein expression in tumors. One possible method is development of an immunohistochemical (IHC) stain for MSP in tumors, and standardized qualitative measurements for the staining intensity. However, MSP is a secreted protein and it is possible that a substantial amount of tumor-produced MSP will be secreted outside of the cells and possibly lost during processing of samples, potentially

leading to inaccurate measurements. Another potential method is measuring the amount of MSP in patient serum or tumor biopsies by enzyme-linked immunosorbent assay (ELISA). While this method has the potential for much more accurate measurements, it is unclear whether the endogenous, inactive forms of MSP produced by the liver will overwhelm the signal from active MSP produced by the tumor. If so, this could result in high background levels and a low signal-noise ratio. However, there are results from two separate studies suggesting that changes in MSP concentrations within human serum can be detected. In the first study, the investigators used glycoproteomic analysis coupled with a 2D-LC-MALDI mass spectrometry system to identify potential biomarkers of osteoarthritic progression (Fukuda 2012). Plasma was isolated from patients with osteoarthritis of the knee, who were determined to be either progressors or non-progressors based on changes in radiographic criteria. The samples were then analyzed to identify proteins whose concentration increased significantly during progression. MSP was identified as one of the biomarkers in this study, demonstrating a significant increase in patients whose disease was progressing (Fukuda 2012). The second study involved detection of polymorphic alleles of MSP that have been identified in genome-wide association studies as significantly linked to susceptibility to inflammatory bowel diseases (Kauder 2013). The investigators were able to demonstrate differences in the MSP protein concentration in patient serum that correlated with their genotype at the polymorphic allele. Taken together, these data suggest that it is possible to detect differences in MSP protein expression in the serum of patients. Our lab

has developed both IHC and ELISA assays for human MSP that are undergoing further development.

Currently, my hypothesis is that overexpression of MSP by tumor cells not only leads to an increase in pro-MSP, but also the cleaved activated MSP due to concomitant expression of high levels of matriptase. Although perhaps the crucial measurement concerns the cleaved, activated form of MSP, unfortunately neither IHC nor ELISA methods are currently capable of specifically detecting active MSP. It may be possible to design an antibody targeting the cleavage site within MSP resulting in the specific detection of pro-MSP. Serum concentrations of MSP would then be measured using an antibody specific for pro-MSP and an antibody which detects both pro- and active MSP. Active MSP concentrations may then be calculated as total MSP subtracted from pro-MSP concentrations. Another possibility would be to perform Western blot analysis on serum samples (under denaturing conditions) for semiquantitative measurements of pro- versus active MSP. While it is clear that the techniques for determining the concentration of active MSP require further development, doing so would allow important questions to be answered, especially for determining whether MSP could serve as a valuable biomarker predicting response to Ron inhibitors. Because the MSP/Ron pathway is not dependent on the RANKL pathway, MSP expression levels may also predict a failed response to RANKL antagonism. My data predict that patients with high levels of MSP may be less responsive to denosumab; Ron inhibitors would be a valuable alternative.

5.4 Markers of bone turnover and their use in clinical trials

Several factors can be involved in determining bone quality, including bone density and qualitative determinants of bone strength such as the rate of bone turnover, the extent of trabecular connectivity, cortical and periosteal bone size, and skeletal morphometry (Wheater 2013). Bone is a metabolically active organ and is constantly being repaired and remodeled throughout an individual's lifetime. In recent years, cellular components of the bone matrix have been identified and categorized as markers of either bone formation or bone resorption. Markers of bone formation are either by-products of active osteoblasts or osteoblastic enzymes. The most widely used markers of bone formation include bone specific alkaline phosphatase (BSAP), osteocalcin, and the amino-terminal propeptide of type I collagen (P1NP) (Vasikaran 2011). The majority of bone resorption markers are degradation products of bone collagen such as carboxy-terminal and amino-terminal cross-linked telopeptides of type I collagen (CTx and NTx, respectively). Another marker of bone degradation is tartrate-resistant acid phosphatase (TRAF5b), an osteoclast-specific enzyme produced by actively resorbing osteoclasts (Vasikaran 2011). Although widely used in research, the diagnostic importance of bone turnover markers remains to be validated, in part due to their analytical and biological variability. Despite this variability, tests to evaluate these biomarkers of bone turnover are often included in clinical trials involving antiresorptive therapies. These tests are thought to be useful in monitoring a patient's response to an antiresorptive treatment, reinforcing patient compliance, and monitoring treatment efficacy before bone

remodeling abnormalities can be evaluated by means such as bone scintigraphy or magnetic resonance imaging (Wheater 2013). It is important to realize, however, that these markers of bone turnover do not themselves control skeletal metabolism and are not disease specific – they reflect the entire skeletal dynamic regardless of the underlying cause. Because of this, these markers can be extremely useful in assessing the biologic action, if not the therapeutic efficacy, of antiresorptive therapy (Black 2007; Bone 2008). While I have observed obvious bone destruction in various models with high MSP expression using imaging, I have not yet measured bone turnover markers. Importantly, I have yet to demonstrate whether these markers are changed upon treatment with Ron inhibitors. The measurement of these markers will become especially important in clinical trials involving Ron inhibitors, as it is a noninvasive technique to determine preliminary efficacy as well as dose selection. Measurements of bone remodeling are currently determined by performing a bone scan involving technetium and single photon emission computed tomography. While this type of imaging is less sensitive, and requires longer time to evaluation, it is still considered the gold standard for measuring bone abnormalities (Wheater 2013). However, new imaging modalities have been and are being developed to accurately image and potentially measure bone turnover. One of the new imaging techniques involves a combination positron emission tomography/computed tomography (PET/CT) scan using the radioisotope sodium fluoride-18 (Na/F^{18}). The fluoride ion is incorporated into metabolically active bone and is capable of detecting bone lesions with a high level of sensitivity

(Schirrmeister 1999; Schirrmeister 1999). What is less clear, however, is whether these imaging technologies (either standard of care or the newly developed technology) are equally sensitive for detecting both osteoblastic and osteolytic lesions. This is a crucial point, as bone lesions in breast cancer are primarily osteolytic and Ron inhibitors are would primarily function to block osteolysis, not osteoblastic activity. I am now performing experiments in the PyMT mouse model utilizing tests to measure markers of bone turnover and Na/F^{18} PET/CT imaging as preclinical experiments to aid in the design of future clinical trials.

5.5 ASLAN002 phase II clinical trial

We are currently in the process of designing a randomized phase II clinical trial in collaboration with ASLAN pharmaceuticals and Bristol-Meyers Squibb (see Appendix and Figure 5.1). This trial will involve breast cancer patients with bone metastases whose bone lesions are not responsive to bisphosphonate therapy. It will likely include an active comparator, though whether this should be zoledronic acid or denosumab has yet to be determined. The primary outcome measure will be the percent change in the bone turnover marker β-CTx from baseline to week 8. This time point may be extended depending upon results at week 8, or on results from any Phase I trial data involving ASLAN002 in combination with standard of care chemotherapy, if applicable. Secondary outcome measures are to be a change in median time to first on-study SRE, and the percentage of participants experiencing on-study SREs. Exploratory outcome measures will include IHC markers for MSP/Ron signaling pathway in tumor

tissue, measurement of MSP in the serum, and progression-free survival. Subgroups will be specified based on the expression of MSP and Ron in primary tumor samples and breast cancer subtype using standard methods of analysis. Analysis of MSP expression will be used to determine whether MSP expression (or levels of expression) can predict a response to ASLAN002. Determining Ron expression will aid in the evaluation of the effect of ASLAN002 on the primary tumor or visceral metastases, if any, and whether this correlates with Ron expression. It is possible that ASLAN002 will have an effect on the primary tumor independently of Ron expression, possibly due to inhibition of another tyrosine kinase target such as Met or Axl. Analysis of collected samples for these kinases would help to determine this. Analysis of breast cancer subtype may give information regarding subtype specific response to Ron inhibitors, and allow the study of MSP-dependent bone destruction among the breast cancer subtypes. Use of a CT scan may allow us to determine whether ASLAN002 has an effect on visceral metastases based on tumor response rate measurements. Finally, analysis of bone turnover markers will allow us to correlate Ron inhibition with the inhibition of tumor-induced bone destruction. The questions answered in this trial will allow us to further understand the mechanism of MSP-dependent bone destruction and, more importantly, determine whether Ron inhibitors represent a safe and effective therapy against bone destruction due to breast cancer bone metastases. This knowledge will also be critical in determining the potential uses of Ron inhibitors not only in breast cancer, but also in other bone metastatic cancers and bone destructive diseases as well.

5.6 MSP and Ron in multiple myeloma

Another cancer type where Ron inhibitors could prove useful is multiple myeloma. Multiple myeloma (MM) is a neoplastic plasma-cell disorder that is characterized by clonal proliferation of malignant plasma cells in the bone marrow microenvironment. It is the second most common hematopoietic malignancy, with an incidence of around 20,000 per year in the United States (Kuehl and Bergsagel 2012). Symptoms of MM primarily include end-organ damage such as lytic bone lesions, anemia, immunodeficiency, and decreased renal function. Myeloma is thought to arise most commonly from a premalignant tumor called monoclonal gammopathy of undetermined significance (MGUS), which then progresses to smoldering myeloma and finally to symptomatic myeloma. MGUS is distinguished from MM by having less intramedullary tumor cell content (<10%). Smoldering myeloma has a stable intramedullary tumor cell content (>10%) but no complications commonly associated with MM. Progression of smoldering myeloma to malignant myeloma is associated with increasingly severe secondary features (lytic bone lesions, anemia) (Kuehl and Bergsagel 2002). Osteolytic lesions develop in approximately 80% of patients with newly diagnosed disease, and around 60% of patients report bone pain (Kyle 2003). Lytic lesions in MM develop due to increased osteoclast activity and decreased osteoblast activity. The mechanism of decreased osteoblast activity is not fully understood but is thought to involve inhibition of the Wnt pathway, which leads to the suppression of osteoblasts. Bone marrow biopsies from MM patients show a correlation between tumor burden, osteoclast number, and resorptive surface but

also demonstrate a decrease in trabecular thickness and low calcification rate with decreased osteoblast numbers indicating a disruption of the balance of bone remodeling (Raje and Roodman 2011).

There are many cytokines and growth factors known to activate osteoclasts in MM. These factors are generated by the mutual interactions of tumor and bone marrow cells and include IL-6, RANKL, and macrophage inflammatory protein 1α (Palumbo and Anderson 2011). Interestingly, the development of osteolytic lesions is not limited to a secondary effect of an MM tumor. Many components of the microenvironment support the propagation of tumor cells and MM is an exquisitely niche-dependent cancer. Several studies using bone-targeted agents have suggested that restoring bone homeostasis may lead to inhibition of tumor growth. Although osteoblasts secrete MM growth factors such as IL-6, they have an overall inhibitory effect on MM proliferation (Yaccoby 2006). For example, inhibition of DKK1, and the resulting relief of osteoblast inhibition, resulted in reduction of tumor growth primarily as an indirect effect via modification of the microenvironment (Fulciniti 2009). Another possible mechanism by which the bone niche (particularly osteoblasts) can suppress MM is that the osteoblast-derived extracellular matrix component decorin, which regulates bone formation and mineralization, induces MM cell apoptosis via p21 activation, and inhibits osteoclastogenesis (Li 2008). Treatment with zoledronic acid also inhibits MM growth, although it is not clear whether this is due to the effects of the bisphosphonate on blocking osteoclast activity, bisphosphonates enhancing the effects of antimyeloma therapy, or whether bisphosphonates have

direct antimyeloma activity. These questions remain unanswered; trial designs always include bisphosphonates as supportive care therapy and data for antitumor activity are gathered in a post hoc analysis (Raje and Roodman 2011). Although osteoclasts are a critical component in the development of bone disease, osteoblasts are also affected in MM and they are known to contribute to the development of osteolysis. It is likely that effective therapeutic strategies to overcome the detrimental osteolysis that occurs in MM patients will target both osteoclasts and osteoblasts, combining bone-anabolic and anticatabolic therapies. Novel agents with dual activity on bone remodeling may not only prevent osteolytic lesions but will also possibly result in improvement of the bone disease. Perhaps more importantly, therapies that restore the balance in bone remodeling in MM may also create an unsupportive microenvironment for MM tumor growth.

Currently, there is very little known about a role for MSP/Ron in MM. Using a publically available cancer data set, I found MSP expression to be significantly higher in MM tumors compared to normal plasma cells (Figure 4.S7A). This is the first evidence suggesting a potential role for MSP in this disease setting and I do not yet know whether Ron is also expressed. I have convincingly shown that the mechanism of MSP-dependent osteolysis is through osteoclast activation and I have also demonstrated that the involvement of osteoblasts is unlikely. I was not able to detect Ron protein expression in an osteoblast cell line and osteoblasts *in vivo* do not stain positively for Ron by IHC (Figures 4.S1G and 4.S1H). I have also shown that Ron TK-/- and STK-/- mice do not have defects in osteoblast

activity as measured by mineral apposition rate or bone formation rate (Figures 4.S1C-F). It is therefore unlikely that inhibition of Ron alone could have the desired effect of modifying the activity of both osteoblasts and osteoclasts. Inhibition of Ron alone would not affect the production of growth factors responsible for fueling tumor growth and suppressing osteoblast activity. However, none of the currently available Ron inhibitors target Ron specifically - they also target the receptor tyrosine kinase Met (Yao 2013). Met is the receptor for hepatocyte growth factor (HGF) and is the only other member of the receptor tyrosine kinase family that includes Ron. Ron and Met are structurally very similar, with 63% amino acid conservation within the kinase domain and 34% overall amino acid identity (Kretschmann 2010). Their ligands, HGF and MSP, are also very similar, with 45% identity. They are both glycoproteins that are secreted as inactive single-chain peptides and need to be proteolytically cleaved to become active. Despite their similarity, however, the ligands and receptors are not interchangeable with each other (Kretschmann 2010).

5.7 Role of HGF/MET in multiple myeloma

While little is known about the function of Ron and MSP in MM, there is a substantial amount of data indicating an important role for Met and HGF in this disease. High levels of both HGF and Met have been observed in MM, allowing for autocrine activation. In addition, bone marrow stromal cells produce HGF, suggesting paracrine stimulation of MM cells within the bone marrow microenvironment can also occur (Borset 1996). Elevated levels of HGF in the

serum and high Met expression by MM tumor cells have both been shown to be associated with poor prognosis in MM patients (Kristensen 2013; Rocci 2014). HGF stimulation of MM cells promotes proliferation, migration, and survival. Additionally, HGF stimulation protects MM cells from apoptosis through RAS mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase protein kinase B (PI3K/PKB) signaling (Derksen 2003). The HGF/Met pathway has also been shown to play an important role in modifying bone metabolism; MET is expressed on osteoclasts and osteoblasts. HGF inhibits bone morphogenetic protein (BMP)-induced osteoblastogenesis and maintains human mesenchymal stem cells in a proliferative, undifferentiated state (Standal 2007). As it is known that MM cells are inhibitory to osteoblasts, and given the high expression of HGF in MM cells, HGF is considered to be one potential osteoblast-inhibiting factor in multiple myeloma.

While the role of HGF in osteoclasts is not entirely clear, it has been shown to substitute for M-CSF in RANKL-induced differentiation and increase the motility of osteoclasts while having no effect on their resorptive activity (Adamopoulos 2006; Gaasch 2006). Additionally, HGF was found in the media of human osteoclast-like cells isolated from bone giant cell tumors grown *in vitro,* suggesting the possibility of paracrine signaling to osteoblasts. Because of this evidence, HGF has been proposed to act as a coupling factor for osteoclasts and osteoblasts (Grano 1996). While an interesting possibility, much of this hypothesis has yet to be investigated.

Given the important role for HGF/Met, and the possible role for MSP/Ron in MM, an inhibitor targeting both of these pathways could potentially disrupt multiple processes involved in MM growth and development of lytic disease. I have shown that inhibition of Ron on osteoclasts can potently block their resorption ability both *in vitro* and *in vivo* (Figures 4.1B and 4.6A). Inhibition of Met in osteoclasts should block their proliferation; however, I have not observed a reduction in the proliferation rate *in vitro* or total osteoclast numbers *in vivo* upon treatment with Ron/Met inhibitors. It is possible that this is the result of the lower IC_{50} for Met vs. Ron in the case of ASLAN002 (Schroeder 2009), but this is not likely the case for OSI296 as it is slightly more Met-selective (Steinig 2013). The effect of those inhibitors on Met activity in osteoclasts and the outcome of Met inhibition will need further study. Inhibition of Met in osteoblasts would be predicted to result in their activation, while inhibition of Ron would presumably have no effect; I have not been able to detect Ron expression in osteoblasts. I did not see an increase in osteoblast activity after treatment with OSI296 or ASLAN002. Experiments utilizing osteoclasts or osteoblasts *in vitro* to test the effects of inhibitors which target Ron or Met preferentially, or inhibitors which target both of these receptors equally, will help to answer questions regarding the effects that these drugs have in these cell types. Despite the lack of evidence for Met inhibition in my *in vivo* experiments, it is important to note that the models I used to test these inhibitors do not express high levels of HGF, and so Met may not be activated in these models. Utilizing mouse models of MM which express HGF, MSP, or both would be a more appropriate way to answer these questions

in vivo. Co-culture *in vitro* experiments may also be required, given the possible role of HGF in paracrine signaling events. Mouse models of MM will help to determine whether MSP or Ron are expressed, and the effect that their expression have on MM tumor growth and osteolysis. Information from patient tumors could be very informative as well. It would be interesting to determine what percentage of MM patient tumors express MSP, and whether MSP expression correlates with disease progression or severity of osteolysis. Determining expression levels of MSP in patient tumors, and potentially in patient sera, will also help to validate the use of MSP overexpression in mouse models of multiple myeloma. Performing these studies in MM will expand our understanding of the role of MSP/Ron in different cancers, and potentially increase the use for Ron/Met inhibitors for treatment. In addition, studying Ron/Met specific or dual inhibitors in models of MM will aid in understanding the interplay of these two pathways and the roles they each have in bone remodeling.

5.8 Role of MSP/Ron in immune-mediated bone loss

A relationship between inflammation and bone disease has long been established in many clinical and experimental models (Spector 1993; Gough 1994; Bernstein 2000; Bultink 2005). It is now understood that the immune system has varied effects on the delicate balance of bone resorption and bone formation, both in physiological and pathological settings (Hardy and Cooper 2009). Indeed, chronic inflammatory diseases are frequently associated with

systemic bone loss and the mechanisms involved are complex and interrelated. These diseases include inflammatory joint disease, inflammatory bowel disease, lung inflammation, renal disease, and diseases affecting nerve and muscle (Hardy and Cooper 2009). While the mechanisms underlying bone loss in these diseases are similar in some respects to one another, they also have distinct components. In addition, specific treatments for these conditions can contribute to the bone loss associated with these diseases. Nevertheless, it is clear that osteoclast activation plays a crucial role in inflammation-associated bone loss.

Rheumatoid arthritis (RA) represents a common example of a systemic inflammatory process, which leads to significant changes in bone metabolism. This inflammatory process targets the articular cartilage, bone at the joint margins, as well as periarticular and subchondral bone (Goldring 2004). During the development of RA, the normally thin synovial lining, composed of fibroblast- and macrophage-like cells, undergoes intense proliferation to form a hyperplastic structure called the pannus. The pannus invades the joint space and destroys the cartilage through inflammatory cytokines, which directly affect chondrocytes, and by degradative enzymes liberated from synoviocytes (Jones 2011; Noss 2008). As the pannus tissue encroaches on periarticular bone, erosion occurs through inflammation-induced osteoclast differentiation and activation (Jones 2011; Takayangi 2009). Various inflammatory mediators expressed within the synovial tissues have the ability to modify the bone remodeling process and promote bone resorption by direct activation of osteoclasts within the pannus. Macrophages and T cells represent the main source of inflammatory cytokines, and the number

of macrophages present at the bone-synovial interface correlates with the degree of bone damage. Additionally, synovial macrophages are thought to serve as the progenitors for osteoclasts within the synovium, though other cell types may be capable of transdifferentiating as well (Takayanagi 2009).

Osteoarthritis (OA) is similar to RA in that it results in pathological changes in the components of the diarthroidal joint. OA is often characterized as a disease of articular cartilage; however, it is becoming clear that changes in the subchondral bone also play a key role in structural destruction of the joint (Suri 2012). Unlike RA, the pathological changes occurring within the joint in OA are thought to occur first, with the associated inflammation acting as a secondary response to the dysfunctional cells and tissues within the joint. This inflammatory response tends to be milder than in RA, although in some patients, the amount of inflammation in OA becomes indistinguishable from that of RA. Similar to RA, where the initial cause of inflammation is unknown, the initial cause of cellular dysfunction in OA is also currently unknown.

5.9 MSP/Ron in inflammation

Ron acts as a crucial regulator of inflammation by inhibiting classical macrophage activation and promoting alternative activation of macrophages. The promotion of alternative macrophages results in the resolution of inflammation and tissue repair (Kretschmann 2010; Wang 2013). Expression of Ron is found on multiple types of resident macrophages, including alveolar macrophages, microglia, peritoneal macrophages, and dermal macrophages (Nanney 1998;

Brunelleschi 2001; Suzuki 2008). Experiments have demonstrated the importance of the MSP/Ron pathway in protection against Gram-positive bacteria. Ron TK-/- mice challenged with *Listeria monocytogenes* showed an increase in bacterial burden and susceptibility to infection. These results suggest that a lack of Ron results in the inability of macrophages to efficiently eliminate intracellular bacteria (Lutz 2003). In models of lung injury, Ron proved to be essential for protection from unregulated inflammation; Ron TK-/- mice displayed increased lung injury due to their inability to downregulate inflammatory cytokines (Lentsch 2007; McDowell 2002). MSP/Ron have also been shown to interact with the acquired immune system, at least in the cancer setting, by suppressing the recruitment of activated CD8+ cytotoxic T cells to the site of metastatic disease (Eyob 2013). However, it is still unclear whether this is due to MSP/Ron signaling in macrophages and the mechanism by which this occurs has yet to be elucidated. It is also not clear whether Ron activation is capable of suppressing CD8+ T cell recruitment in other disease settings.

5.10 MSP/Ron in inflammatory arthritis

Given the role of the MSP/Ron pathway in osteoclast activation and inflammation in various settings; it is plausible that this pathway may also function in inflammatory diseases. In types of arthritis associated with bone destruction, such as RA and OA, macrophages within the synovial lining play a key role in the inflammatory process. Interestingly, MSP has been shown to be expressed in synovial lining cells by *in situ* hybridization (Fukuda 2012). This

evidence suggests that there may be a source of MSP produced within the inflamed joint, though it is not known whether this source is from macrophage-like or fibroblast-like cells, both of which compose the synovial lining. The accumulation of macrophages could potentially provide a source of matriptase that could cleave and activate pro-MSP (Bhatt 2007). In addition, matriptase is highly expressed in the synovial cartilage of OA patients compared to normal cartilage, where it acts to stimulate collagenolysis during pathogenesis (Milner 2010). Activation of MSP could then cause Ron activation in osteoclasts located within the pannus, thereby leading to bone resorption. Finally, MSP was identified as a potential biomarker in the plasma of patients with OA whose plasma levels correlated with disease progression (Fukuda 2012). The presence of synovial inflammation often associated with OA is believed to be a secondary phenomenon related to the destruction of cartilage and release of breakdown products into the synovial fluid. However, the changes that occur in the synovium are often indistinguishable from that of patients with RA (Pelletier 2001). It would be interesting to determine whether MSP is also expressed in the joints of RA patients and whether MSP plasma concentrations correlate with RA disease progression. In addition, while it is likely, matriptase expression has yet to be demonstrated in cartilage from RA patients. Thus, Ron inhibitors may also be useful to combat inflammatory arthritis.

5.11 Chondrocytes in inflammatory arthritis

Chondrocytes are another important cell type involved in inflammatory arthritis. Under normal physiologic conditions, these cells express various proteolytic enzymes such as matrix metalloproteinases (MMPs), which mediate the very low levels of matrix turnover responsible for cartilage remodeling (Van Osch 2009). In pathological conditions such as RA or OA, chondrocytes increase the production of these enzymes considerably, resulting in aberrant cartilage destruction (Pelletier 2001; Aigner 2002). I have found for the first time that Ron is expressed in chondrocytes (Figure 4.S1G); however, its function in these cells is still completely unknown. One important factor produced at high levels by chondrocytes during pathogenesis is MMP13 (Chiu 2007). MMP13 (also known as collagenase 3) is the most potent of the enzymes responsible for the degradation of type II collagen, the primary collagen type in cartilage (Li 2011; Troeberg 2012; Martel-Pelletier 1996). While it is not known whether Ron activation in chondrocytes leads to MMP13 expression, I have found that PyMT tumor cells overexpressing MSP have 2 to 3 fold higher MMP13 expression compared to PyMT control tumors (unpublished data). It would be very interesting to determine whether MSP stimulation of Ron in chondrocytes leads to MMP13 production, which could implicate a role for this pathway not only in bone destruction, but also in the cartilage destruction that occurs in inflammatory arthritis. Similar to MMP13, chondrocytes also produce high levels of the inflammatory mediator IL-6 (Pelletier 2001), another cytokine produced downstream of MSP/Ron signaling in some cell types (Banu 1996). It is therefore

possible that Ron activation in chondrocytes could lead to the production of IL-6, which has the potential to not only contribute to the overall inflammatory process but to bone destruction as well; IL-6 is capable of inducing osteoclastogenesis (Kudo 2003). Using the chondrocyte-specific Cre expressing mouse (Col2a1-Cre) along with the osteoclast-specific Cre mouse (Cathepsin K-Cre) that we now have in the lab could be useful in differentiating between the effects of Ron in osteoclasts and the potential role of Ron in the chondrocytes in various disease models.

While the role of the MSP/Ron pathway in RA and/or OA is an interesting possibility, there are many questions that need to be investigated. The levels of MSP protein present within the arthritic synovial fluid of both RA and OA patients needs to be determined. More importantly, the ratio of pro-MSP compared to cleaved, activated MSP needs to be determined, as arthritic joints may have more activated MSP due to high matriptase. If MSP is indeed expressed by cells within the synovial lining, these cells need to be identified. This would be particularly interesting as the production of systemic MSP is primarily in the liver. The role of Ron in these diseases also needs to be elucidated. There are many models used to study both RA and OA. If Ron has an important role in osteoclast activation in these diseased settings, it is very likely that the Ron TK-/- mouse would be resistant to arthritis-induced bone loss. It would also be interesting to test the efficacy of Ron inhibitors in this setting. Ron inhibition could block not only bone destruction, but may potentially also block some of the inflammation that occurs. Osteoclast activation in response to inflammatory cytokines such as

IL-6 and TNFα has not been tested; it would be interesting to see if treatment could reduce resorption, even in the absence of MSP. Factors capable of activating osteoclasts are often analyzed together. It would be interesting to determine which pathways are driving the bone destruction in RA/OA. Whether MSP/Ron have a role in macrophage function within the setting of RA and OA also needs to be determined. Due to existing evidence describing important roles in immune response, specifically in macrophages, it is likely that there is a role; however, the function of MSP/Ron within the immune system may be context dependent. While its initial role is to stimulate the immune system, its most important role is thought to be in shutting down the immune response once infection or injury has been resolved. It is possible that in RA/OA the MSP/Ron pathway is expressed in an attempt to control the rampant inflammation occurring. However, the expression of the pathway may inadvertently lead to bone destruction, and possibly increased cartilage destruction, which only enhances the inflammatory response in these diseases.

In conclusion, I have demonstrated that MSP/Ron functions as an important pathway not only in bone biology, but also in cancer-induced bone destruction. I have shown that MSP/Ron are capable of regulating many important functions within the osteoclast and may be an important player in many pathological diseases that influence bone remodeling. Finally, I have demonstrated that Ron inhibitors successfully block tumor-induced bone destruction and osteoporotic bone loss. Future work will define Ron signaling in the osteoclast and the potentially therapeutic effects of Ron blockade. It will also

help to determine the applicability of the Ron pathway in other cancers, pathologic bone diseases, and the feasibility of using these inhibitors in various clinical applications.

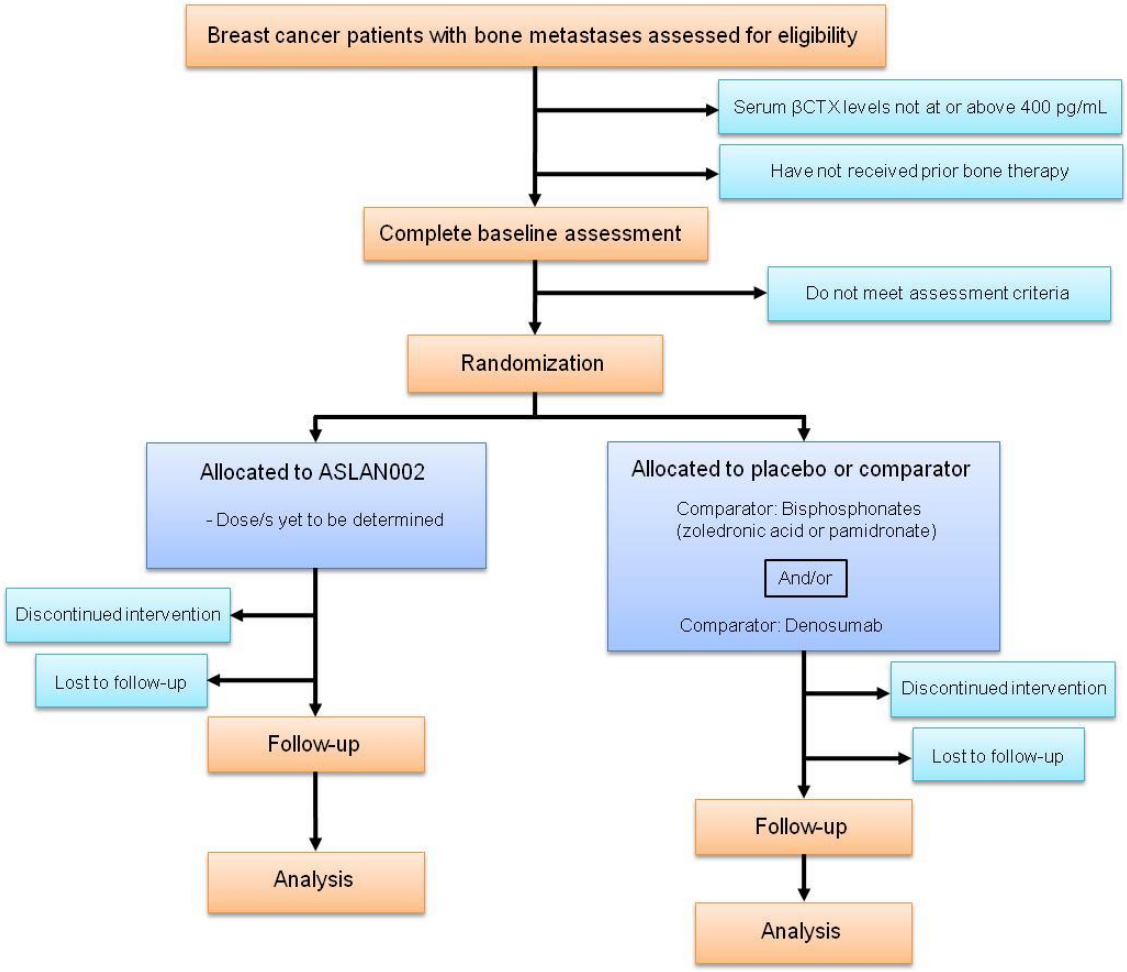


Figure 5.1 Flow chart for a two-arm, parallel, randomized clinical trial with ASLAN002

5.12 References

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APPENDIX

PHASE II RANDOMIZED CLINICAL TRIAL OF ASLAN002

FOR BREAST CANCER BONE METASTASIS

A.1 Introduction

The most common site of metastasis in breast cancer patients is bone, which occurs in about 80% of patients with advanced disease. A characteristic feature of these metastases is their osteolytic nature, which causes remarkable bone loss, fractures, severe pain, hypercalcemia, and other detrimental effects. The Macrophage Stimulating Protein (MSP), its receptor RON, and the protease that activates MSP, MT-SP1, are highly expressed in up to 20% of breast cancer patients. These patients exhibit increased metastasis to lung, liver, brain, and bone, with bone being the most frequent site of metastasis. Treatment with a RON inhibitor, ASLAN002, successfully inhibits the development and progression of osteolytic lesions caused by breast cancer cells in an animal model. In addition, ASLAN002 is currently in a phase I clinical trial and has demonstrated a relatively safe and reversible toxicity profile which can be easily monitored (NCT01721148). Taken together, these data suggest that Ron could be a potentially useful therapeutic target in breast cancer metastasis to bone for the treatment of osteolytic lesions, particularly when the patients' tumor expresses the RON ligand, MSP.

There is a great need for new therapies for metastatic cancers which have progressed on or are unresponsive to current therapy. There are no approved RON targeted agents in standard clinical use. In the population of subjects with advanced breast cancer metastatic to bone whose tumors are unresponsive to standard of care, in addition to the relatively safe toxicity profile of ASLAN002 in

a clinical study, the potential for benefit from ASLAN002 outweighs the potential risks for toxicity.

A.2 Primary hypothesis

Our central hypothesis is that patients with metastatic breast cancer unresponsive to bisphosphonate therapy who are treated with ASLAN002 have lower markers of bone resorption compared to placebo.

A.3 Purpose

The purpose of this study is to identify the safety and efficacy of ASLAN002 in a randomized, phase II study of subjects with advanced breast cancer which has metastasized to bone.

A.4 Treatment arms and justification

Placebo versus ASLAN002 (administered orally)

- Justification: Patients eligible to enroll in the trial have failed the FDA-approved standard-of-care bisphosphonates and placebo is therefore justified
- Standard of care medications for metastatic disease (other than bone) will be allowed

A.5 Primary and secondary endpoints

A.5.1 Primary outcome measures

- Percent change in β–CTX from baseline to week 8

A.5.2 Secondary outcome measures

- Change in F^{18}/Na PET SUV signal from baseline to week 8
- Overall response rate in visceral metastases determined by RECIST criteria at week 8
- Overall safety profile of ASLAN002 (adverse events, labs) at week 8
- Bone pain (use of analgesic medications) at week 8
- Percentage of participants experiencing on-study skeletal related event(s) (SREs) at week 8
- Median time to first on-study SRE

Skeletal Related Events (SREs) are defined as a:

- Pathologic bone fracture such as nonvertebral and vertebral compression fractures
- Spinal cord compression identified by clinical exam documented by X-ray evidence
- Surgery to bone both curative and prophylactic
- Radiation therapy to bone, including palliative, therapeutic, or prophylactic

- Hypercalcemia of malignancy, defined as a corrected serum calcium > 12 mg/dl (3.00 mmol/l) or a lower level of hypercalcemia which is symptomatic and which requires active treatment other than rehydration

A.5.3 Exploratory outcome measures

- To explore biomarkers that are potentially predictive of biological response to ASLAN002, including IHC markers for MSP/Ron signaling pathway activity in tumor tissue and serum
- Progression-free survival

A.5.4 Rationale for selection of outcome measures

Use of percent change in markers of bone turnover as a surrogate outcome is appropriate for the following reasons (Brown 2003; Coleman 2005; Fizazi 2009; Leeming 2006; Lein 2007; Lipton 2007; Piedra 2013):

- The continuous variable of bone turnover assays allows for maximum power in the study in addition to providing meaningful data in a short time period
- Changes in biochemical markers of bone turnover correlate significantly with a change in fracture risk (SRE) and predict long-term response to bone therapy
- Response in bone turnover markers during therapy predicts patient overall survival

- FDA approval of bone targeted therapeutics is given after evidence is provided by phase III trials using the surrogate outcome of median time to first on-study SRE and/or total number of on-study SREs. Overall survival and progression-free survival are often included as exploratory end points and are usually reserved for phase III trials with a longer follow-up time period.

A.6 Study design

A.6.1 Design summary

This is a randomized phase II study of ASLAN002 administered orally to subjects with advanced or metastatic breast cancer for whom the standard of care is either ineffective or inappropriate. Subjects will be enrolled in cohorts of 92 patients to either placebo or ASLAN002 on a once daily schedule with a dose of 600 mg for 8 weeks.

A.6.2 Duration of study

The intervention and follow-up is expected to last at least 12 weeks with treatment lasting 8 weeks and follow-up lasting at least 4 weeks. Subjects may discontinue from treatment because of disease progression, unacceptable toxicity, or at the subject's request. Subjects who withdraw from treatment before 8 weeks will be asked to participate in a final visit 4 weeks after discontinuation of therapy. The recruitment phase is anticipated to take a year.

A.6.3 Subgroup specification

- Expression of Ron in primary tumor samples (immunohistochemical staining) (positive/negative)
- Expression of MSP in primary tumor samples (immunohistochemical staining) (positive/negative)
- Breast cancer subtype (ER+, HER2+, and triple negative)

A.7 Recruitment

A.7.1 Eligibility inclusion criteria

- Female patients ≥ 18 years of age
- Able and willing to give written informed consent
- Histologically confirmed Stage IV breast cancer with at least one bone metastasis radiologically confirmed (including radiography, computed tomography (CT), PET scan, PET/CT scan, magnetic resonance imaging, bone scan, or skeletal survey) that have either progressed on standard therapy or for whom standard therapy is not known.
- Life expectancy of ≥ 3 months
- Prior anticancer treatments are permitted (i.e., chemotherapy, radiotherapy, hormonal, or immunotherapy)
- Prior bone therapies will be permitted. Three weeks must have elapsed between prior bone therapy and the initiation of the study therapy
- Patients unresponsive to bone therapy as indicated by levels of serum β-CTx ≥ 400 pg/mL

- Archived primary tumor tissue available in paraffin-embedded blocks or slides
- Ability to comply with visits/procedures required by the protocol

A.7.2 Eligibility exclusion criteria

- Patients unable to swallow or take anything orally
- Patients with a serum creatinine > 3 mg/dL (265 µmol/L) or calculated (Cockcroft-Gault formula) creatinine clearance (CrCl) < 30 mL/min CrCl = ({[140-age (years)] x weight (kg)}/ [72 x serum creatinine (mg/dL)]) x 0.85
- Patients with corrected serum calcium ≤ 8.0 mg/dL and > 11.6 mg/dL
- Women who are pregnant (with a positive pregnancy test prior to study entry) or lactating patients
- Women of childbearing potential unwilling or unable to use effective methods of birth control (e.g., oral contraceptives or implants, IUD, vaginal diaphragm or sponge, or condom with spermacide)
- Patients treated with systemic investigational drug(s) and/or device(s) within the past 30 days or topical investigational drugs within the past 7 days.
- Patients currently receiving bisphosphonates. Bisphosphonates must have been discontinued ≥ 3 weeks prior to randomization
- Patients currently receiving Denosumab. Denosumab must have been discontinued ≥ 2 weeks prior to randomization
- Patients currently treated with radiotherapy. Radiotherapy must be discontinued ≥ 3 weeks prior to randomization

- Patients currently treated with any drugs known to affect the skeleton (e.g., calcitonin, mithramycin, systemic glucocorticoids, or gallium nitrate) within 2 weeks prior to randomization
- History of diseases with influence on bone metabolism, such as Paget's disease, osteogenesis imperfecta, and primary or secondary hyperthyroidism within 12 months prior to study entry
- Patients with known symptomatic brain metastasis. Subjects with controlled brain metastasis (no radiographic progression at least 4 weeks following radiation and/or surgical treatment and no neurological signs or symptoms) will be allowed
- History of allergy to ASLAN002 (BMS-777607) or chemically related compounds

A.7.3 Discontinuation of subjects from treatment

Subjects must discontinue study treatment for any of the following reasons:

- Withdrawal of informed consent (subject's decision to withdraw for any reason)
- Any clinical adverse event, laboratory abnormality or intercurrent illness which, in the opinion of the investigator, indicates that continued participation in the study is not in the best interest of the subject
- Disease progression as determined by RECIST criteria

All patients who discontinue study treatment should comply with protocol specified follow-up procedures as stated below.

A.8 Procedures

Blood is drawn and urine collected at specified time points for bone marker analysis and labs (fasting morning sample). Blood samples will be collected on all subjects at screening, prior to dosing on Day 1, and at time points indicated. Samples taken during screening will be evaluated according to eligibility criteria for laboratory values. In addition, levels of β-CTx in the serum will be measured to determine whether the patient is unresponsive to prior bone therapies (≥ 400 pg/mL).

- Required to take oral placebo or ASLAN002 daily
- Imaging for a Na/F^{18} PET/CT scan and X-ray

A.8.1 Data and specimens to be collected

- Levels of bone markers for bone turnover in patient serum
- PET/CT signal for tumor growth and bone turnover
- Laboratory test assessments (hematology, serum chemistry, urinalysis, pregnancy test)
- Tissue sections of the primary tumor (archived)
- Patient demographics and baseline characteristics
- Vital signs
- Physical examination and measurements (height, weight and ECOG performance status)
- Patient medical history
- Breast cancer subtype (determined by standard histology)

A.8.2 Criteria for evaluation

- Safety outcome measures: All patients who receive treatment will be evaluated for safety. Safety assessments will be based on medical review of adverse event reports and the results of vital sign measurements, physical examinations, and clinical laboratory tests. Toxicity will be evaluated according to the NCI Common Terminology Criteria for Adverse Events (CTCAE v. 3.0).
- Efficacy measures: Determine metastatic tumor response [Time Frame: From baseline to 8 weeks (+/- 7 days)]. Tumor response will be determined for all subjects with measurable disease as defined by the RECIST criteria. Computed tomography assessments will be made every 4 weeks or more frequently if indicated. Changes from baseline tumor size determined by RECIST criteria and the percent change from baseline in the sum of the longest diameters in target lesions will be determined. A maximum of 5 lesions per organ and 10 lesions in total should be identified as target lesions to be measured. The target lesions should be representative of all involved organs. The baseline sum of the longest diameter of target lesions will be used as the reference by which to characterize the objective tumor response. Tumor response will be determined as follows:
    - Complete Response (CR): Complete disappearance of all tumor lesions.

- Partial Response (PR): Decrease, relative to baseline, of 30% or greater in the sum of the longest diameter of all target lesions. Additionally, patients must not meet the criteria for progressive disease.
- Stable Disease (SD): Failure to meet criteria for complete or partial response, in the absence of progressive disease.
- Progressive Disease (PD): At least 20% increase in the sum of the longest diameter of all target lesions or the appearance of any new lesions.

- Progression-free survival (PFS) will be defined as the time between the first dose of study therapy and the date of progression or death. A subject who dies without reported prior progression will be considered to have progressed on the date of death. For those who remain alive and have not progressed, PFS will be censored on the date of last tumor assessment. Assessment of the Eastern Cooperative Oncology Group (ECOG) Performance Score [Time Frame: From baseline to 8 weeks]. ECOG Performance Score has 4 grades. 0 = Fully active, able to carry out all predisease activities; 1 = Restricted in strenuous activity but ambulatory and able to carry out work of light or sedentary nature; 2 = Ambulatory and capable of all self-care but unable to carry out work activities. Active about 50% of waking hours; 3= Capable of limited self-care, confined to bed/chair more than 50% of waking hours; 4 = Completely disabled,

cannot carry on self-care. Totally confined to bed/chair. Outcome will be summarized as median score for participants at baseline and at 8 weeks.

- Bone turnover will be assessed by measuring the levels of serum beta-CTX, TRAP-5b, P1NP, and BSAP [Time Frame: From baseline to 8 weeks (+/- 2 days)]. Outcome is given as percent change from baseline to 8 weeks. Additional measures of bone lesion activity will be assessed by Na/F^{18} positron emission tomography (Na/F^{18} PET)[Time Frame: From baseline to 8 weeks]. Outcome is given as change from baseline or standardized uptake value (SUV) of target lesions. SUV is quantified according to standardized uptake value representing a region of interest corrected for the injected activity and for patient weight or lean body mass.
- Bone pain will be measured as the use of analgesic medications according to the Analgesic Score Scale [Time Frame: From baseline to 8 weeks]. The outcome is given as the median score for the participants at baseline and 8 weeks of treatment.

  The analgesic score used for this study is modified from the Radiation Therapy Oncology Group (RTOG) analgesic score scale. The scale represents type of medication administered from 0 to 4 where 0 = None

  1. = Minor analgesics (aspirin, NSAID, acetaminophen, propoxyphene, etc.)

  2. = Tranquilizers, antidepressents, muscle relaxants, and steroids

  3. = Mild narcotics (oxycodone, meperidine, codeine, etc.)

  4. = Strong narcotics (morphine, hydromorphine, etc.)

- Pharmacogenomic/predictive measures: MSP expression, RON expression, and breast cancer subtype are the potential predictive biomarkers. Tumor material from all subjects will be evaluated during screening to determine the presence of these proteins or the proteins which define each breast cancer subtype (Estrogen Receptor (ER), Progesterone Receptor (PR), and Human Epidermal Growth Factor Receptor 2 (HER2)) performed by immunohistochemistry (IHC) and described by both percent positivity and overall strength of stain (weak, moderate, strong). A patient sample will be considered positive for the expression of ER, PR, Ron, and/or MSP if ≥ 1% of the tissue is positive by IHC, ≤ 1% will be considered negative for the expression of these markers. A patient sample will be considered positive for HER2 if the sample has an intensity score of ≥3+, ≤2+ will be considered negative for HER2 expression.

A.8.3 Expected adherence to protocol/dropout problems,
and steps taken to handle them

Study drug compliance is assessed at each in-person study visit by comparing the expected versus actual consumption of study medicine. Each subject will bring in all remaining study drug in the original bottles to each follow-up visit. The study coordinator will count and record the number of remaining tablets. At each visit, a new supply sufficient to carry the subject until their subsequent visit will be dispensed. Protocol adherence will be a major focus of

in-person study visits. Episodes of non-adherence will be reviewed twice weekly, and a report will be provided to the investigators and DSMB quarterly.

A.8.4 Premature discontinuation of study therapy

Participants who prematurely discontinue treatment with the study therapy regimen will remain in the study and follow the Schedule of Events. Study drug may be prematurely discontinued/ terminated for any participant for life-threatening reactions. The study drug may also be prematurely discontinued for any participant if the investigator believes that the treatment is no longer in the best interest of the subject, if the subject is judged noncompliant, or due to safety concerns.

A.8.5 Blinding/masking

We will employ a double blind study design to reduce the risk of bias. Intervention assignments will be carried out via simple blocked randomization using a block size of 2, after eligible participants are screened. A matched placebo will be used in the control group. This placebo will have as close to same color, weight, shape, texture, taste, odor and dissolution properties as the study medication (ASLAN002 600 mg) as possible. Active drug and placebo will be tested for matching closeness by an independent panel of observers. The study drug and matched placebo will be delivered orally and presented in plain containers without marks or identifiers other than the codes which will be only decipherable with the key. Each participant will have a unique drug code that will stay with her for the duration of the trial. Emergency unblinding procedures will

be conducted under specific emergent conditions where unblinding is absolutely necessary (examples: child ingestion, serious adverse reaction).

A.8.6 Randomization scheme and blinding

With a sample size of 92, we will use simple block randomization, using a block size of 2. This will ensure equal numbers in both arms (placebo versus active drug). Factors associated with outcome include: histopathologic subtypes, receptor status, grading, DNA classification level, and MSP expression level. We anticipate at least 10% in each of the above categories. Thus, simple randomization would ensure somewhat balanced enrollment of patients with various prognostic factors and there is no need for stratification.

This is a single center study. Randomization will be done after screening, on site. A computer generated list will be kept in a password protected encrypted computer on site. A back up of the list will be kept on a separate password protected encrypted computer. The randomization scheme for the first 20 subjects is attached. The list is created in Excel, using the rand() command. With a block size of 2, there are 2 permutations: A (active drug, placebo), B (placebo, active drug). If the random number is < 0.5, we will choose permutation A. If the random number is ≥ 0.5, we will choose permutation B.

A.8.7 Justification of sample size

The Power and Sample Size (PS) software (DuPont, Vanderbilt) was used to calculate the required sample size for a two-sample t-test assuming a two-

sided alpha of 0.05 and a power of 90% across a range of possible clinical differences and standard deviations of the response variable, percent change in β–CTX from baseline to 8 weeks (Table A.2). Because this is an early phase trial, there are very little existing data to inform sample size calculations. The starting points for our inputs were based on estimates of the change in β–CTX in breast cancer patients from baseline to week 25 in the phase II trial described by Fizazi and colleagues. In this study, the median (IQR) percent change in serum CTX from baseline to 25 weeks was 34.6% (22.3%) for breast cancer patients receiving IV bisphosphonates (3).

The final sample size was chosen based on an assumed detectable difference of 15% and a standard deviation of 20%, resulting in an estimated 76 required patients total (3). In order to account for possible dropouts, the sample size was inflated by 20% to arrive at a final total sample size of 92 patients, with equal allocation of 46 patients each to the placebo and active treatment arms.

As the DSMB will only review safety aspects of the trial, no interim efficacy analyses are planned and therefore no adjustments were made to the overall alpha for the trial.

A.8.8 Data analysis and collection techniques

Data will be collected by trained study nurses and clinicians using structured data collection instruments. Abstracted data will be double-entered into a secure research database such as REDCap. All discrepancies will be resolved by an independent reviewer. Characteristics of patients assigned to

placebo and to investigational treatment will be compared using two-sample t-tests for normally distributed continuous variables, Wilcoxon rank-sum tests for non-normally distributed continuous variables, and with chi-square and Fisher's exact tests, as appropriate, for categorical variables. Additional descriptive analyses will be performed comparing patients who were and were not randomized, as well as patients who remained on-study to those who were lost to follow-up, if necessary.

A.8.9 Primary analysis

The primary endpoint and some secondary endpoints will be assessed at 8 weeks for all study patients. As there will be no variability in follow-up time, these endpoints will be evaluated using simple two-sample t-tests and chi-squared tests. These analyses will be conducted in accordance with the intention-to-treat principle. Secondary analyses will evaluate the per-protocol population. The time to first on-study SRE will be evaluated using Kaplan-Meier product-limit survival methods and Cox proportional hazards, if necessary based on the performance of the randomization scheme. An extended Cox model will be used to account for deaths within a competing risks framework. Missing data will be handled using multiple imputation under the assumption that values are missing at random (MAR).

Subgroup analyses will be performed to evaluate efficacy in patients with and without RON expression in their primary tumor sample, in patients with and

without MSP expression in their primary tumor sample, and by breast cancer subtype (ER+, HER2+, triple negative).

A.8.10 Interim monitoring plan

The Data and Safety Monitoring Board (DSMB) will review safety data quarterly during planned DSMB Data Review Meetings. Data for the planned safety reviews will include, at a minimum, a listing of all reported Adverse Events and Serious Adverse Events. The DSMB will be informed of an Expedited Safety Report in a timely manner.

In addition to the prescheduled data reviews and planned safety monitoring, the DSMB may be called upon for ad hoc reviews. The DSMB will review any event that potentially impacts safety at the request of the protocol investigator. After careful review of the data, the DSMB will make recommendations regarding study conduct and/or continuation.

A.8.11 Temporary halt for emergency safety review

A temporary halt in enrollment will be implemented if an ad hoc DSMB safety review is required. In the event that the study temporarily halts enrollment, no new subjects will be consented or start on therapy, and subjects already on study therapy will continue on therapy unless they are the focus of the DSMB review. Subjects in the screening phase of the study may continue to undergo minimal risk procedures (e.g., blood tests); all other procedures should be

deferred. Randomization will not occur until the DSMB review is complete. The health authorities will be notified of any halt in enrollment.

A.9 Definitions

A.9.1 Adverse event (AE)

An adverse event (AE) is defined as any untoward or unfavorable medical occurrence associated with the use of a drug in humans, whether or not considered drug related. An adverse event may include any unfavorable or unintended sign (including an abnormal laboratory finding), symptom, or disease temporally associated with the use of an investigational product.

A.9.2 Suspected adverse reaction (SAR)

A suspected adverse reaction (SAR) is any adverse event for which there is a reasonable possibility that the investigational drug caused the adverse event. For the purposes of safety reporting, ‘reasonable possibility’ means there is evidence to suggest a causal relationship between the drug and the adverse event. A suspected adverse reaction implies a lesser degree of certainty about causality than adverse reaction, which means any adverse event caused by a drug.

A.9.3 Unexpected adverse event

An adverse event or suspected adverse reaction is considered “unexpected” if it is not listed in the investigator brochure or is not listed at the specificity or severity that has been observed; or, if an investigator brochure is

not required or available, is not consistent with the risk information described in the general investigational plan. “Unexpected” also refers to adverse events or suspected adverse reactions that are mentioned in the investigator brochure as occurring with a class of drugs or as anticipated from the pharmacological properties of the drug, but are not specifically mentioned as occurring with the particular drug under investigation.

A.9.4 Serious adverse events (SAE)

An adverse event or suspected adverse reaction is considered “serious” if, in the view of the investigator, it results in any of the following outcomes:

- Death
- A life-threatening event: An AE is considered “life-threatening” if, in the view of the investigator, its occurrence places the subject at immediate risk of death. It does not include an AE that, had it occurred in a more severe form, might have caused death.
- Inpatient hospitalization or prolongation of existing hospitalization
- Persistent or significant incapacity or substantial disruption of the ability to conduct normal life functions
- Important medical events that may not result in death, be life threatening, or require hospitalization may be considered serious when, based upon appropriate medical judgment, they

may jeopardize the subject and may require medical or surgical intervention to prevent one of the outcomes listed above.

A.9.5 Causality or relatedness definition

The relationship, or attribution, of an adverse event to the study drug will initially be determined by the investigator and recorded on the appropriate AE/SAE form. The relationship of an adverse event to study therapy regimen or procedures will be determined using the descriptors and definitions provided in Table A.3.

A.9.6 Severity or intensity definition

The severity of adverse events experienced by the study subjects is according to the criteria set forth in the National Cancer Institute's Common Terminology Criteria for Adverse Events Version (CTCAE). This document (referred to herein as the NCI-CTCAE manual) provides a common language to describe levels of severity, to analyze and interpret data, and to articulate the clinical significance of all adverse events.

Adverse events will be graded on a scale from 1 to 5 according to the following standards in the NCI-CTCAE manual:

Grade 1 = mild adverse event.

Grade 2 = moderate adverse event.

Grade 3 = severe and undesirable adverse event.

Grade 4 = life-threatening or disabling adverse event.

Grade 5 = death.

Adverse events will be collected from the time of the first protocol mandated procedure until the study completion, or until 30 days after the subject prematurely withdraws from the study.

A.10 Collection and recording of adverse events

A.10.1 Collection period

Adverse events will be collected from the time of first protocol mandated procedure, until subject completes study participation or until 30 days after subject prematurely withdraws (without withdrawing consent) or is withdrawn from the study.

A.10.2 Collecting adverse events

Adverse events (including SAEs) may be discovered through any of these methods:

- Observing the subject.
- Questioning the subject in an objective manner.
- Receiving an unsolicited complaint from the subject.
- In addition, an abnormal value or result from a clinical or laboratory evaluation can also indicate an adverse event.

A.10.3 Recording adverse events

Throughout the study, the investigator will record adverse events and serious adverse events on the appropriate AE/SAE form regardless of the

relationship to study therapy regimen or study procedure. All adverse events must be reported on the appropriate adverse events form within 5 business days of the learning of the adverse event(s). Once recorded, an AE/SAE will be followed until it resolves with or without sequelae, or until the end of study participation, or until 30 days after the subject prematurely withdraws or is withdrawn from the study, whichever occurs first.

A.11 Reporting of serious adverse events

A.11.1 Reporting of serious adverse events to sponsor

Timely reporting of adverse events is required. Investigators must report all serious adverse events, regardless of relationship or expectedness to the IRB an sponsor within 2 days of discovering the event. For serious adverse events, all requested information on the AE/SAE should be provided to the DSMB. However, unavailable details of the event should not delay submission of the known information. As additional details become available, the AE/SAE form should be updated and submitted.

A.11.2 Final report

A final study report will be provided to health authorities of all adverse events classified as:

- Serious, expected, suspected adverse reactions
- Serious and not a suspected adverse reaction
- Pregnancies not reported as serious adverse events.

Note that all adverse events (not just those requiring 2 day reporting) will be reported in the final report.

A.11.3 Expedited reporting within 15 calendar days

The sponsor must notify the appropriate health authorities and the investigators as soon as possible, or within 15 calendar days if the adverse event is classified as one of the following:

- Serious and unexpected suspected adverse reaction. Expedited reports are to include, in an aggregate analysis, specific events that occur more frequently in the investigational drug than in a concurrent or control group.
- Any findings from other studies that suggest a significant risk in humans exposed to ASLAN002. This includes findings from animal or *in vitro* testing that suggest a significant risk in humans exposed to the drug. Ordinarily, such a finding would result in a safety-related change in the protocol, informed consent, or other aspects of the overall conduct of the trial, will be reported.

A.11.4 Expedited reporting within 7 calendar days

The sponsor must notify the appropriate health authorities and the investigators as soon as possible, or within 7 calendar days, of any unexpected fatal or immediately life-threatening suspected adverse reaction.

A.11.5 Reporting of adverse events to IRBs

Investigator will report adverse events, including expedited reports, in a timely fashion to the local IRB in accordance with applicable regulations and guidelines.

A.12 Data management

A.12.1 Privacy protections

A computer-generated list will be kept in password protected encrypted computer on site. A back up of the list will be kept on a separate password protected encrypted computer. Only the randomization administrator will have access to this list.

A.12.2 Confidentiality precautions

Participant identifiers will be stored separately from the coded, participant data. All data that will be transferred or transported outside of the institution will be encrypted and stored on password protected encrypted computers. There will be no photos, medical images, or recording (voice or video) obtained

A.12.3 Collaborator communications

We will use eRoom™ to provide a “digital office” to support secure, confidential communication and collaboration among study investigators. The software is Web–based and uses an office metaphor of rooms that may contain folders, documents, task lists, calendars, and task oriented databases. This web-

based application will provide researchers secure access to research data even if not inside the study area. All data stored on eRoom will be de-identified.

A.12.4 Study site

Huntsman Cancer Institute High Risk Breast Cancer Clinic will serve as the research site for the investigation. The High Risk Breast Cancer Clinic is under the direction of Dr. Saundra S. Buys.

A.12.5 Network

The Huntsman Cancer Institute coordinates its network infrastructure and security with the Health Sciences Campus (HSC) information systems at the University of Utah. This provides Huntsman Cancer Institute with effective firewall hardware, automatic network intrusion detection, and the expertise of dedicated security experts working at the University. Network equipment includes three high-speed switches and two hubs. User authentication is centralized with two Windows 2003 domain servers. Communication over public networks is encrypted with virtual point-to-point sessions using secure socket layer (SSL) or virtual private network (VPN) technologies, both of which provide at least 128 bit encryption. eRoom™ and other web applications use the SSL protocol to transmit data securely over the Internet. Direct access to Huntsman Cancer Institute machines is only available while physically located inside the Huntsman Cancer Institute offices, or via a VPN client. All network traffic is monitored for

intrusion attempts, security scans are regularly run against our servers, and our IT staff is notified of intrusion alerts.

A.13 Strengths and limitations of overall design

A.13.1 Strengths

This study is strengthened by the use of randomization, blinding, and use of a placebo comparator group.

A.13.2 Limitations

The study is short term and is not designed to address survival points (progression free survival). In addition, the short study follow-up and small sample size are not adequate to accurately reflect effect of the drug on skeletal events. It is likely that MSP expression can function as a biomarker for response to ASLAN002. If MSP expression is rare in breast cancer patients, using an unselected patient population may result in a lack of overall efficacy even if the drug was efficacious in the MSP expressing subpopulation. In addition, this is a single center investigation enrolling a very specific patient population, and our results may not be generalizable to other populations.

Table A.1 Chart and events schedule

Table A.1: Flow Chart and Events Schedule

Event	Screening		Treatment									Follow-up
	Day -30 to Day -8	Day -7 to Day -1	Day 7	Day 14	Day 21	Day 28	Day 35	Day 42	Day 56	End of treatment	Day 84
Sign protocol specific informed consent form	X										
Medical History	X										
Physical Measurements	X	X			X			X		X	X
Physical Examination	X	X	X	X	X	X	X	X	X	X	X
Performance status and vital signs	X	X	X	X	X	X	X	X	X	X	X
Serum chemistry testing	X	X	X	X	X			X			X
Urinalysis w/micro	X		X					X			X
CBC with differential	X	X	X	X	X	X	X		X	X	X
Coagulation profile	X				X			X			X
Serum HCG	X	X						X			X
Archived tumor tissue block or slides	X										
Na/F18 PET/CT		X				X			X		
X-ray (as indicated)						X			X		
Blood sample for bone turnover markers	X	X	X	X	X	X	X	X	X	X	X
Monitor for serious/nonserious adverse events, concomitant medications	Collected from time of informed consent										

Table A.2. Sample size estimates for a two-arm parallel group design with two-sided alpha of 0.05 and power of 90%

Detectable difference	SD	n per group	total n
20%	5%	3	6
	10%	6	12
	20%	22	44
	30%	48	96
	40%	85	170
15%	5%	4	8
	10%	10	20
	20%	38	76
	30%	85	170
	40%	150	300
10%	5%	6	12
	10%	22	44
	20%	85	170
	30%	190	380
	40%	337	674
5%	5%	22	44
	10%	85	170
	20%	337	674
	30%	758	1516
	40%	1346	2692

Table A.3 Descriptions and definitions to determine causality of adverse events

Code	**Descriptor**	**Relationship**
Unrelated Category		
1	**Unrelated**	**The adverse event is clearly not related**
Related Categories		
2	**Possible**	**The adverse event has a reasonable possibility to be related; there is evidence to suggest a causal relationship.**
3	**Definite**	**The adverse event is clearly related.**

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